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Applicants:	Andrew VAILLANT et al.
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For:	ANTIVIRAL OLIGONUCLEOTIDES TARGETING HIV
Art Unit:	1648
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DECLARATION UNDER 37 C.F.R. SEC. 1.132

I, Jean-Marc Juteau, do hereby declare and state as follows:

1. I received the degrees of Bachelor (B.Sc.) of Biology from Montreal University in 1985, Master (M.Sc.) of Microbiology and Immunology from Montreal University in 1988, and Doctor of Philosophy (Ph.D.) of Microbiology and Immunology from Laval University in 1991 .
2. My academic background and experiences in the field of the present invention are listed on the enclosed *curriculum vitae.*
3. I am a founder since 1999 of REPLICor Inc. and Senior Vice President since 2002.
4. I am an author of several scholarly publications as listed in my enclosed *curriculum vitae.*

5. I am an inventor in the present application; I have read and am thoroughly familiar with the contents of U.S. Patent Application Serial No. 10/661,099, entitled "ANTIVIRAL OLIGONUCLEOTIDES TARGETING HIV", including the claims.

6. I have also read and understood the latest Official Action from the PTO dated December 8, 2005. In this Office Action, certain claims 1, 2, 14-20 and 26-32 were rejected for lack of enablement under 35 U.S.C. §112, first paragraph.

7. The following experiments had been performed in Jan-Feb 2006 (SIV model) and Sept 2004 (Friend Leukemia Virus), under the supervision of Andrew Vaillant (inventor on this invention) and myself, to obtained results with a Simian Immunodeficiency Virus model showing the anti-retroviral activity of sequence independent oligonucleotides of the present invention in a non-human primate. In addition, experiments have also been accomplished in a Friend Leukemia Virus model, demonstrating the anti-retroviral activity of sequence independent oligonucleotides of the present invention.

   The following experiment was conducted to evaluate the anti-retroviral activity of sequence independent oligonucleotides in a non-human primate.

   Background of the model: The infection of a rhesus macaque with the (Simian Immunodeficiency Virus) SIV is considered to be the animal model to most closely approximate the infection of humans with HIV, SIV, HIV-1 and HIV-2 are retroviruses showing extensive homology in their genomes. The rhesus macaque SIV model closely mimics the progression of human HIV infection. Furthermore, the similarity between SIV and HIV pathogenesis in rhesus

macaques and humans provides a useful model in the macaque for studying HIV pathogenesis. Finally, chronic infection of macaques with SIV eventually results in a disease state whose symptoms closely resemble AIDS in humans and the same laboratory markers can be used to monitor this disease progression. Thus, the SIV model is considered an excellent model for the development of AIDS vaccines.

References: Charkrabarty, 1987, Nature 328:543-547; Hirsh, 2000, Advances Pharmacol. 49:437-477; Chung et al. 2005, Clin. Diagn. Lab. Immunol. 12:426-435; Van Rompay, 2005, AIDS Review 7:67-83 and Hu, 2005, Current Drug Targets – Inf. Disorders 5:193-201 (copy of references enclosed with the present Declaration).

To establish the suitability of an oligonucleotide (ON) as a therapy for HIV infection in humans, we tested its ability to reduce serum viral titers in a macaque chronically infected with SIV.

Materials and Methods

Compound and dose preparation

The sodium salt of a phosphorothioated (PS) 40mer randomer ON, REP 2006, was prepared under GMP compliant manufacturing protocols and prepared for administration by dissolution in sterile normal saline. Dose concentrations were formulated based on the assumption that the infected macaque maintained a constant weight of 4.5 kg throughout the study (which is consistent with empirical observations in this model). A dose-escalation regimen was employed, with the subject transitioning to the next highest dose at the end of a two week period.

Route of administration

Compound was administered by continuous intravenous administration through a cannula inserted in the jugular vein. Infusion rates were controlled by a programmable pump according to the following schedule:

10cc/h for 2h → 0.5cc/h for 10h → 10cc/h for 2h → 0.5cc/h for 10h.

Compound was prepared so that individual subjects received the indicated daily mg/kg dose during the two daily 2h infusions at the elevated infusion rate. The animal continued to receive the same concentration of compound between 2h infusions but at $1/20^{th}$ of the more rapid infusion rate to ensure that cannulas were kept patent throughout the course of the study. Pump reservoirs were changed every 24h to ensure constant dosing.

SIV infection and titer determination

Throughout the study, serum SIV titers were monitored each week using a commercial bDNA SIV assay (performed at Bayer Reference Labs). At the beginning of the study, the macaque was infected with SIV_{mac251} by bolus IV injection. The infection was allowed to proceed until titers had stabilized for at least 3 weeks prior to initiation of treatment. Treatment began the week after the macaque on the study had demonstrated stable titers for at least three weeks.

Results: As shown in Table 1, the macaque showed a continuous drop in viral titer during the 11 week treatment, demonstrating the effectiveness of a sequence independent PS-ONs in lowering the SIV titers in the infected macaque when administered by daily continuous infusion.

Table 1
Reduction in serum titer in a REP 2006 treated, SIV-infected macaque

WEEK	REP 2006 DOSE (total mg/kg/day)	SIV serum titer (copies / ml)
1	0	1479300 (pre-treatment titer)
2	1	2784300
3	1	1245900
4	2	1070400
5	2	826304
6	4	648507
7	4	581487
8	6	477427
9	8	639965
10	8	575179
11	10	548428
12	10	427217

Conclusion: Administration of daily doses of REP 2006 resulted in a continuous drop in viral titer in a SIV model, thus demonstrating the anti-retroviral activity *in vivo* of the sequence independent oligonucleotides of the present invention.

A further experimentation was conducted to evaluate the anti-retroviral activity of sequence independent oligonucleotides in mice.

Background of the model: The Friend Leukemia Virus (FLV) is an immunosuppressive retrovirus such as HIV. Although the FLV model is not as close to HIV as is the SIV model, it is a well established model for studying genetic resistance to infection (Hasenkrug, 1997, Proc. Natl. Acad. Sci. USA, copy enclosed).

Materials and Methods: Mice received 10mg/kg of REP 2006 (the same sodium salt formulation as described above) by a once daily 500ul bolus IP injection on days -2, -1, 0, 1, 2, 3, 4, 5.

Mice received FLV innoculum on day 0 by IV injection.

Spleens were harvested on day 6 and infection was monitored by fluorescence assisted cell sorting (FACS) to detect the percentage of infected splenocytes using mAb 34 which detects the FLV gag protein only expressed on the surface of infected splenocytes.

Results: As shown in Table 2, treated mice showed a 2.5 fold reduction of FLV infected splenocytes compared to untreated animals. These data support the hypothesis that REP 2006 could be used as an effective treatment to treat retroviral infections as shown. A t-test to determine the significance of the difference between the placebo and REP 2006 treated groups yielded a P-value of 0.0085, indicating that the effectiveness of REP 2006 was statistically significant.

Table 2
Summary of statistical data (percentage of infected splenocytes)

Parameter	Placebo (5% dextrose)	10mg/kg/day REP 2006
Mean	29.3	11.27
N	4	4
Std. dev.	6.99	6.22
Maximum	22.8	6.3
Minimum	38.7	19.3
Lower 95% CI	18.17	1.37
Upper 95% CI	40.43	21.179

Conclusion: treated mice showed a 2.5 fold reduction of FLV infected splenocytes compared to untreated animals, thus demonstrating the anti-retroviral activity *in vivo* of the sequence independent oligonucleotides of the present invention.

8. The results presented above and produced according to the teaching of the present invention clearly proves that that the present invention have clinical relevance and in addition, that the *in vitro* results disclosed in the present application do not diverge from *in vivo* responses. The anti-retroviral activity of the sequence independent oligonucleotides of the present invention is demonstrated in non-human primates and in a Friend Leukemia Virus model.
9. I hereby declare that all statements made herein of my own knowledge are true, and that all statements made on information and belief are believed to be true, and that these statements were made with the knowledge that willful false statements and the like so made are punishable by a fine or imprisonment, or both (18 U.S.C. Sec. 1001), and may jeopardize the validity of the application of any patent issuing thereon.

Signed  Dated: June 06, 2006

Jean-Marc Juteau

Curriculum vitae

JEAN-MARC JUTEAU, Ph.D

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Age: 42

Status: Married, three kids

Language spoken and written: French and English

EXPERIENCE

01-2002 - today

Senior Vice-President and Founder, REPLICor Inc., Laval.
Biopharmaceutical company developing antiviral and anticancer drugs.

Responsibilities:

- Science development.
  Day to day contact with CSO, scientific input.
- In charge of intellectual property portfolio.
  Patent writing, strategy, management.

02-1999 – 01-2002

CEO and founder, REPLICor Inc., Laval.

Responsibilities:

- Science development
- In charge of financing
  Instrumental in raising $2.5M in equity and loan
- In charge of licensing and contract agreement
  Negotiation of licenses and contracts with universities

02-1996 to 02-1999

Officer, Office of Technology Transfer, McGill University, Montreal.

Responsibilities:

- Agreement management and negotiation
  License, research, option, confidentiality, material distribution.
- Spin-off company projects
  Set-up of spin-off company, contact with investors, business plan.

03-94 to 02-96

Product Manager, Iso Tech Design, Laval
Company developing and marketing micro-environments for pharma applications.

Responsibilities:

- Microbiology quality control..

- Distributor formation
  Contacts: Baxter Health Care, VWR, Khulman Tech., E.S.I. FluFrance, Liberty Clean Rooms, Millipore.

91 à 10-93

Director and Co-founder, DIAGNOGENE inc., R&D in biotechnology, Ste-Foy
Responsibilities: Financial and research administration, representation.

RESEARCH TRAINING

09-92 à 11-93

Post-doctoral scientist, **INRS-santé**, Pointe-Claire
Project: In-vitro mutagenesis of 4-chlorobenzoate dehalogenase in *Pseudomonas sp.* CBS3.

08-91 à 09-92

Post-doctoral scientist, **Institut de Recherches Cliniques de Montréal**
Project: Cloning et characterization of a cardiac specific transcription factor.

11-90

Training in molecular modeling, Department of Molecular and Cell Biology, **University of Connecticut.**

05-88 to 06-88

Workshop on DNA technologies: Sequence and in-vitro mutagenesis, **University of North-Carolina**, Chapel Hill, NC.

EDUCATION

87-91

Doctorate (Ph.D)., Microbiology and Immunology, **Laval University.**
Molecular biology, epidemiology and structure-function analysis of the ROB-1 ß-lactamase.

85-87

Master (M.Sc)., Microbiology and Immunology, **Montreal University and Hôtel-Dieu Hospital.**
Granulocytar function in recurrent vaginitis.

82-85

Bachelor (B.Sc.)., Biology, **Montreal University.**

BOARD MEMBERSHIP

2005- today

Member of the Montreal Life Science Committee.

2004- today

President of the Alumni Association of Montreal Clinical Research Institute.

SCHOLARSHIP, AWARD and PRIZES

Industrial Design Prize 1995 from the Design Institute (received in team for a micro-environment)
Institut National de la Recherche Scientifique (INRS) Fellowship, 1992-93.
Medical Research Council (MRC)Fellowship, 1992.
Fonds de la Recherche en Santé du Québec (FRSQ) Studentship, 1989-90-91.
Fonds pour la Formation des Chercheurs et l'Aide à la Recherche (FCAR) Studentship, 1988-89.
Canlab Prize from l'Association des Microbiologistes du Québec, 1989.

AUTHORSHIP

Patent filings:	20
Scientific articles:	10
Posters and oral presentations:	30

Vaillant A, Juteau JM, Lu H, Liu S, Lackman-Smith C, Ptak R, Jiang S. Phosphorothioate oligonucleotides inhibit human immunodeficiency virus type 1 fusion by blocking gp41 core formation. Antimicrob Agents Chemother. 2006 Apr;50(4):1393-401.

Kocisko DA, Vaillant A, Lee KS, Arnold KM, Bertholet N, Race RE, Olsen EA, Juteau JM, Caughey B. Potent antiscrapie activities of degenerate phosphorothioate oligonucleotides. Antimicrob Agents Chemother. 2006 Mar;50(3):1034-44.

Moaddel R, Price GB, Juteau JM, Leffak M, Wainer IW. The synthesis and initial characterization of an immobilized DNA unwinding element binding (DUE-B) protein chromatographic stationary phase. J Chromatogr B Analyt Technol Biomed Life Sci. 2005 Jun 25;820(2):197-203.

Sylvestre M, Sirois M, Hurtubise Y, Bergeron J, Ahmad D, Shareck F, Barriault D, Guillemette I, Juteau Sequencing of Comamonas testosteroni strain B-356-biphenyl/chlorobiphenyl dioxygenase genes: evolutionary relationships among Gram-negative bacterial biphenyl dioxygenases.Gene. 1996 Oct 3;174(2):195-202.

Ahmad D, Fraser J, Sylvestre M, Larose A, Khan A, Bergeron J, Juteau JM, Sondossi M. Sequence of the bphD gene encoding 2-hydroxy-6-oxo-(phenyl/chlorophenyl)hexa-2,4-dienoic acid (HOP/cPDA) hydrolase involved in the biphenyl/polychlorinated biphenyl degradation pathway in Comamonas testosteroni: evidence suggesting involvement of Ser112 in catalytic activity.Gene. 1995 Apr 14;156(1):69-74.

Juteau JM, Billings E, Knox JR, Levesque RC. Site-saturation mutagenesis and three-dimensional modelling of ROB-1 define a substrate binding role of Ser130 in class A beta-lactamases. Protein Eng. 1992 Oct;5(7):693-701.

Maclean IW, Slaney L, Juteau JM, Levesque RC, Albritton WL, Ronald AR. Identification of a ROB-1 beta-lactamase in Haemophilus ducreyi. Antimicrob Agents Chemother. 1992 Feb;36(2):467-9.

Juteau JM, Cote S, Levesque RC. Systematic site-saturation mutagenesis of ROB-1 beta-lactamase: efficiency of T4 polymerase and oligonucleotide synthesis. Biotechniques. 1991 Oct;11(4):460-2.

Juteau JM, Sirois M, Medeiros AA, Levesque RC. Molecular distribution of ROB-1 beta-lactamase in Actinobacillus pleuropneumoniae. Antimicrob Agents Chemother. 1991 Jul;35(7):1397-402.

Juteau JM, Levesque RC.Sequence analysis and evolutionary perspectives of ROB-1 beta-lactamase. Antimicrob Agents Chemother. 1990 Jul;34(7):1354-9.

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Sequence of simian immunodeficiency virus from macaque and its relationship to other human and simian retroviruses

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Because of the growing incidence of AIDS (acquired immune deficiency syndrome), the need for studies on animal models is urgent. Infection of chimpanzees with the retroviral agent of human AIDS, the human immunodeficiency virus (HIV), will have only limited usefulness because chimpanzees are in short supply and do not develop the disease. Among non-human primates, both type D retroviruses and lentiviruses can be responsible for immune deficiencies. The D-type retroviruses[1–3], although important pathogens in macaque monkey colonies, are not satisfactory as a model because they differ in genetic structure and pathophysiological properties from the human AIDS viruses[4,5]. The simian lentivirus, previously referred to as simian T-cell lymphotropic virus type III (STLV-III), now termed simian immunodeficiency virus (SIV) is related to HIV by the antigenicity of its proteins and in its main biological properties, such as cytopathic effect and tropism for CD4-bearing cells[6–9]. Most importantly, SIV induces a disease with remarkable similarity to human AIDS in the common rhesus macaques, which therefore constitute the best animal model currently available[10]. Natural or experimental infection of other monkeys such as African green monkeys or sooty mangabeys has not yet been associated with disease[8,9,11]. Molecular approaches of the SIV system will be needed for biological studies and development of vaccines that could be tested in animals. We have cloned and sequenced the complete genome of SIV isolated from a naturally infected macaque that died of AIDS. This SIV_{MAC} appears genetically close to the agent of AIDS in West Africa, HIV-2 (ref. 12), but the divergence of the sequences of SIV and HIV-2 is greater than that previously observed between HIV-1 isolates[13].

We have previously shown that probes derived from the HIV-2 genome could detect viral DNA in SIV_{MAC} infected cells[14]. Cloned subgenomic fragments of HIV-2, representing *gag-pol*, *env* or long terminal repeat (LTR) sequences, were used to screen, in low stringency conditions, a genomic library of HUT-78 cells infected by SIV_{MAC} isolate Mm142-83 (ref. 6). Rhesus monkey Mm142-83 became infected with SIV *in utero* and had constant health problems until her death at ~2 years of age with immunodeficiency and lymphoproliferative syndrome[6,15]. The nucleotide sequence of one SIVmac142 clone, λSIV1, that hybridized with all HIV-2 subgenomic probes, was determined (Fig. 1). The clone λSIV1 contains one integrated provirus, lacking only the first 257 base pairs (bp) of the left LTR; the right LTR is complete and followed by 7 kilobases (kb) of cellular DNA. Biological activity of this clone (monitored by reverse transcriptase assay) was shown in HUT 78 cells at day 12 after transfection (Y. Naidu, Y. Li, H. Kester and G. Jaenel, unpublished results).

The genome of SIV mac is 9,643 nucleotides long (in its RNA form). The organization of its open reading frames, 5′ LTR-*gag*-*pol*-central region-*env*-F-3′LTR (Fig. 2), is typical of a lentivirus[16] and similar in its basic features to that of the HIVs[12]. The only remarkable difference in the genetic organizations of SIV, HIV-1 and HIV-2 resides in the open reading frame (ORF) we termed X, absent in HIV-1[17] and differently positioned relative to ORF R in SIV and HIV-2.

Nucleotide sequence comparison of SIV, HIV-1 (ref. 17) and HIV-2 (ref. 12) reveals considerable homology between SIV and HIV-2. These two viruses share about 75% overall nucleotide sequence homology, but both of them are only distantly related to HIV-1 with about 40% overall homology.

The restriction map of the SIV_{MAC142} isolate, derived from the nucleotide sequence of λSIV1 is similar to those previously established by molecular cloning and Southern blotting[18,19] for the remarkably related viruses apparently originating from West Africa and isolated from African green monkeys (STLV-III_{AGM}), or from healthy individuals (human T-cell lymphotropic virus type IV, HTLV-IV). Of 31 sites, 27 are conserved (87%) between λSIV1 and the STLV-III/HTLV-IV consensus map. This finding is surprising when one considers the variability of HIV-1 isolates and the fact that less than 30% of the sites are conserved between λSIV1 and the ROD isolate of HIV-2 (ref. 12), from which HTLV-IV is serologically indistinguishable. Other analyses[19,20] strongly suggest that the original isolates of STLV-III_{AGM} and HTLV-IV are laboratory acquired contaminants with SIV_{MAC251} which was obtained from the same closed colony as the isolate we studied[20]. To support this idea, comparison of SIV_{MAC}LTR with published partial LTR sequences of STLV-III_{AGM} or HTLV-IV[19] reveals a homology of over 98%.

The SIV LTRs are 831 bp long, and by alignment to those of

Table 1 % Homology between retroviral proteins

a	*Gag*	*Pol*	*Env*							
SIV_{MAC} compared with			EGP	TMP	*F*	*Q*	*R*	*X*	*Tat*	*Art*
$HIV\text{-}2_{ROD}$	87	83.6	75.4	74.1	59.8	73.4	70.3	85.7	59.2	61.0
$HIV\text{-}1_{BRU}$	57.3	59	34.0	49.1	45.7	37.8	57	—	48.1	33.3
$HIV\text{-}2_{ROD}$ compared with										
$HIV\text{-}1_{BRU}$	57.7	59.4	39.4	44.8	37.7	34.6	52.2	—	42.8	44.8
b	$HIV\text{-}2_{ROD}$	$HIV\text{-}1_{BRU}$	$HIV\text{-}1_{ELI}$	$HIV\text{-}1_{MAL}$	VISNA	EIAV	MPMV		HTLV-I	RSV
SIV_{MAC}	83.6	59	58.2	59.2	42.7	44.5	34		34.7	35.7
$HIV\text{-}2_{ROD}$	—	59.4	61.6	59	43.7	43.8	37.8		34.8	35.9
$HIV\text{-}1_{BRU}$	59.4	—	94.4	92	41.9	42.2	36.4		33.3	34.5

Alignments were performed using the program NUCALN[31] with default parameters. The number indicates the % of amino-acid identity in the aligned domains, that is excluding the regions of insertion/deletion. *a*, Comparisons of all proteins of HIV-1 (ref. 17), HIV-2 (ref. 12) and SIV. *b*, Comparisons of *pol*-encoded proteins of different retroviruses. $HIV\text{-}1_{ELI}$ and $HIV\text{-}1_{MAL}$ are Zairian isolates of HIV-1 (ref. 13). EIAV: equine infectious anaemia virus[32]. MPMV: Mason Pfizer monkey virus[4]. HTLV-I: Human T-cell leukaemia virus type I (ref. 33). RSV: Rous sarcoma virus[34].

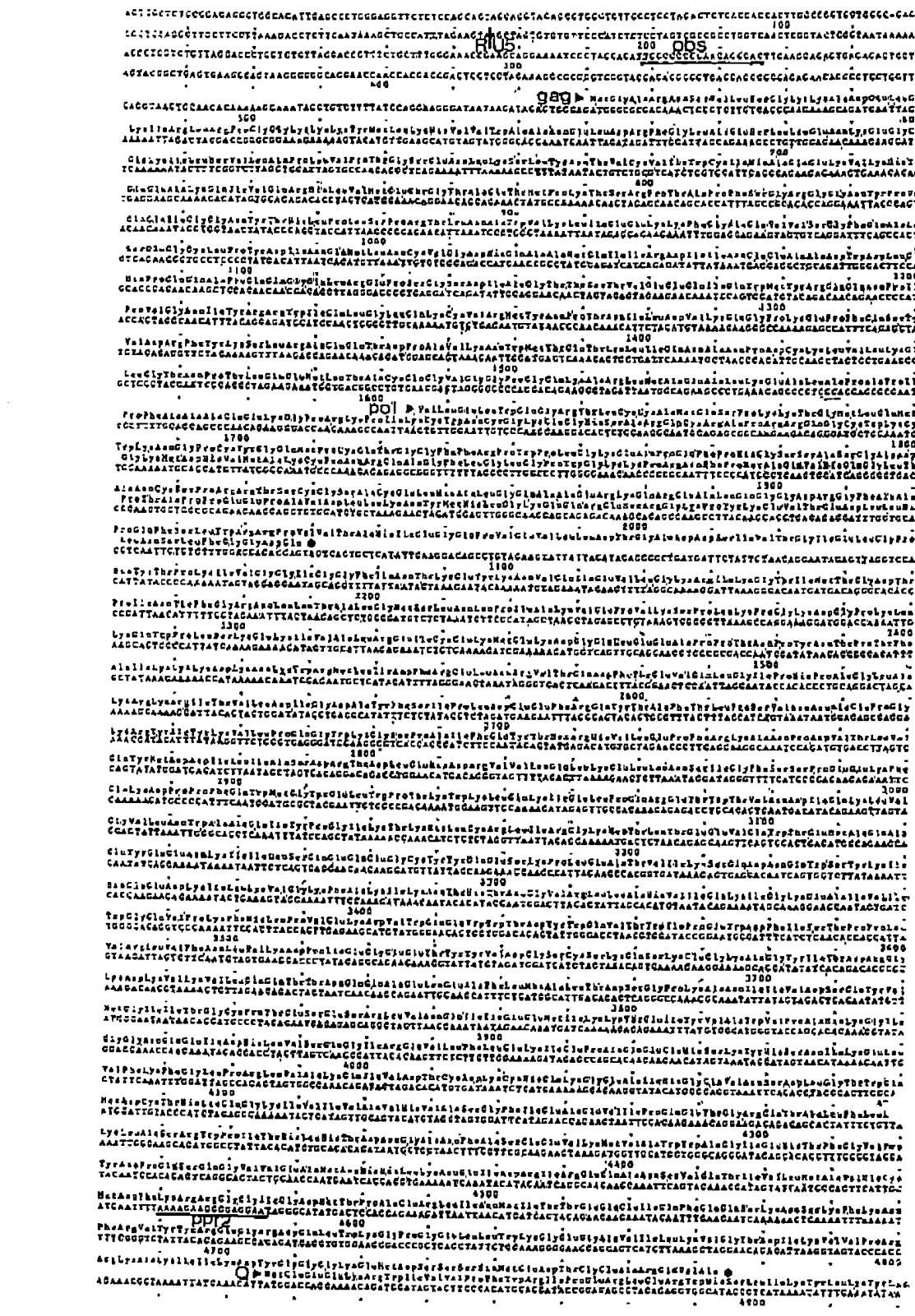
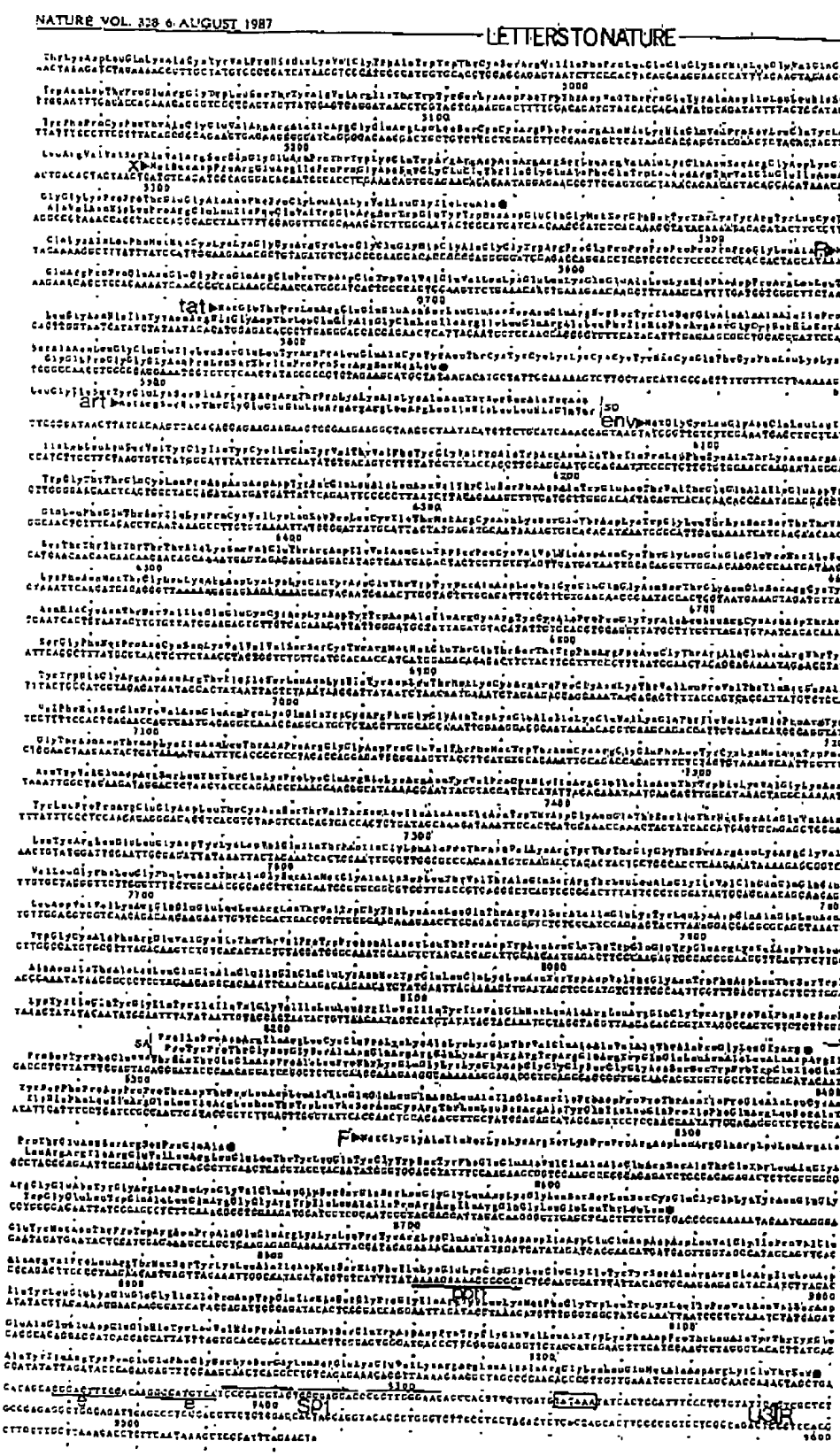


Fig. 1 Left and opposite, the complete nucleotide sequence of the SIV_{MAC} genome. The 9,643 nucleotides are numbered from the cap site to the polyadenylation site and shown together with the deduced amino-acid sequences. PBS, primer binding site complementary to lysine tRNA. Ppt1 and ppt2 are the two copies of the polypurine tract. In *U3* are shown the two enhancer-like sequences (e, underlined, ref. 35), the Sp1-like binding sites (Sp1, overlined) and the promoter (TATAAA, boxed). The genes are translated from the first AUG of the ORF, except for *pol* where the whole ORF is translated. Filled circles, termination codons. SD and SA indicate the splice donor and acceptor of the intron of the *tat* and *art* genes. The in-frame stop codon found in the *env* gene at position 8,298 is indicated by three asterisks. **Methods.** Molecular cloning of SIV proviral DNA was carried out with Sau3A partial digest of DNA from HUT78 cells infected with isolate 142-83 (ref. 6) and λEMBL3 arms (supplied by Vector Cloning Systems). Plaques were screened *in situ* using nick-translated subgenomic fragments of HIV-2 (ref. 14) as probes. Stringency of hybridization, 50 °C in 5× SSC and washing 50 °C in 2× SSC. Sequencing of proviral DNA from λSIV1 was carried out using standard M13 shotgun and dideoxy nucleotides procedures as already described[16].


tat
art
env
rev
SD
SA
pol
LTR

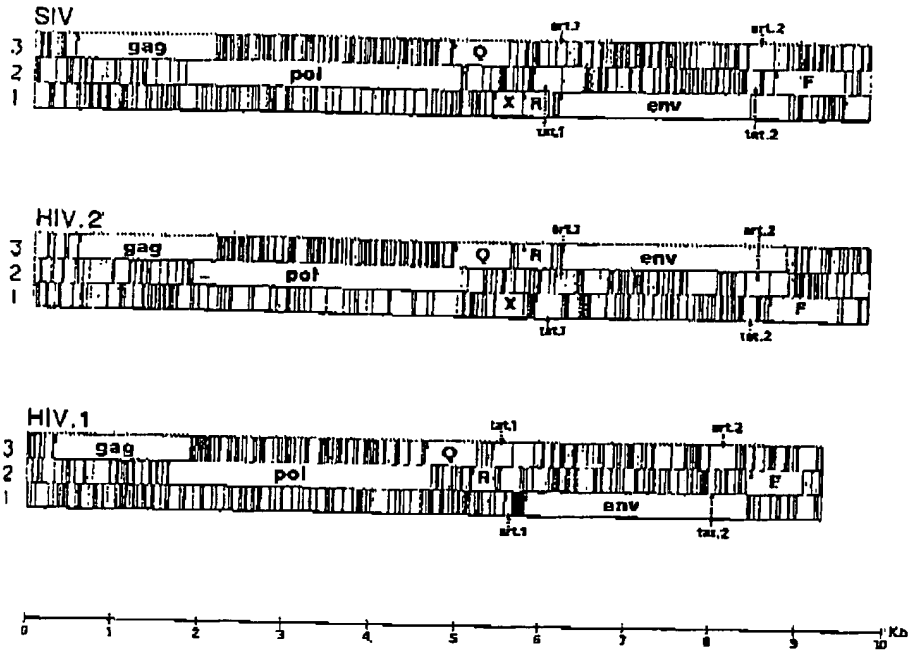


Fig. 2 Genomic organization of SIV compared to HIV-1 (BRU isolate, ref. 17) and HIV-2 (ROD isolate, ref. 12). Vertical bars represent the stop codons in the three reading frames. Arrows indicate the potential AUG initiator codon of each open reading frames. Tat1&2 and art1&2 designate the open reading frames containing the first and second coding exons of the *tat* and *art* genes.

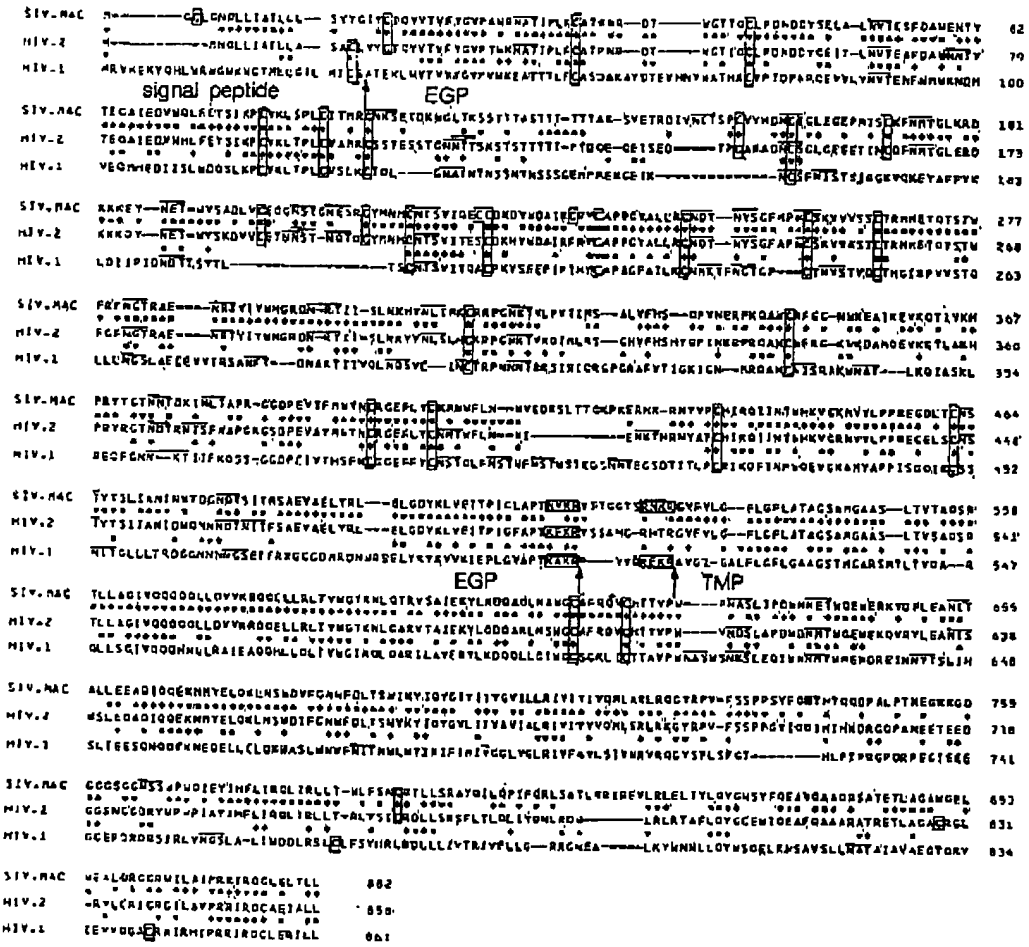


Fig. 3 The alignment of the SIV_{MAC}, $HIV\text{-}2_{ROD}$ (ref. 12) and $HIV\text{-}1_{BRU}$ (ref. 17) envelope proteins. Gaps were introduced to optimize the alignment. Asterisks indicate amino-acid identity. Potential cleavage sites are boxed and shown by arrows. Cysteines are boxed, potential *N*-glycosylation sites are overlined. The filled square in the SIV_{MAC} sequence corresponds to an in-frame nonsense codon. EGP, external glycoprotein; TMP, transmembrane protein.

HIV-2, the length of the internal domains of the SIV LTR have been estimated as: *U3*, 514; *R*, 175; *U5*, 142. The nucleic-acid homology with HIV-2 LTR is 66% in *U3*, 70% in *U5* and 95% in *R*. This important level of LTR homology is associated with conservation of transcription signals and secondary structures common to HIV-1 and HIV-2 LTRs[12].

The *gag* precursor of SIVmac has a calculated relative molecular mass (M_r) of 58.9 K, comparable with the mass of the p55 antigen, estimated from SDS gels; this precursor is likely to be processed into the proteins designated p16 (amino terminus, calculated M_r = 15.3 K), p27 (major core protein) and p12 (carboxy-terminal, nucleic-acid binding protein[11,21,22]). The N-terminal extremity of the $p27^{gag}$ of SIV isolated from a macaque (*Macaca nemestrina*) at the Washington Primate Research Center (MnIV/WRPC) was sequenced previously[23] and only 1 difference out of 23 amino acids is observed with SIV_{MAC142} (Val5 > Ile). In the same region, 2 differences have been found with HIV-2 and 10 (with 1 insertion) with HIV-1.

The SIV *pol* ORF (*pol* protein product, total M_r = 119.6 K) probably encodes the p64 and p53 antigens (reverse transcriptase and endonuclease[22]. The SIV *env* ORF could encode the gp160, gp120 and gp32 antigens[11,21,22]: the precursor (p env), the external glycoprotein (EGP) and the transmembrane protein (TMP). The calculated M_r of non-glycosylated products are p-env, 101 K; EGP, 60 K; TMP, 40.7 K; the numbers of potential *N*-glycosylation sites are: EGP, 22; TMP, 4 (Fig. 3).

An in-frame nonsense codon interrupts the *env* ORF at nucleotide 8,298 in clone λSIV1. Strikingly, the same stop codon (TAG) was found at exactly the same position in the envelope of an integrated genome of HIV-2, but not (replaced by CAG) in complementary DNA derived *in vitro* from viral RNA of the same isolate[12]. Such stop codons experimentally introduced after the hydrophobic part of the TMP (in the so-called cytoplasmic tail) in Rous sarcoma virus do not impair retroviral replication *in vitro*[24]. In SIV, as in HIV-2, the stop codon marking the beginning of the *env* ORF matches with the splice donor site of the first coding exons of the *tat* and *art* genes. Also, the in-frame stop matches with the splice acceptor site for the second coding exons of these genes. The part of the *env* ORF that corresponds to the second intron of the *tat* and *art* regulating genes is thus exactly delimited by two stop codons. The existence of these stop signals could influence (or result from) the differential splice that gives rise alternatively to *tat* and *art* (in a regulating-like phase) or *env* (in a structural-like phase) and may modulate the viral gene expression and pathogenicity, as recently observed for visna virus (ref. 25 and R. Vigne, personal communication). Such codons may also account for the variations in the reported size[12,20,27] of the TMP for HIV-2 or SIV: gp40, gp36 or gp32 (calculated M_r: total TMP, 41K; from cleavage site to stop, 24K).

Comparisons of the proteins of SIV with those of HIV-1 and 2, summarized in Table 1*a*, quantify the relatedness of these viruses. Although SIV and HIV-2 appear closely related, their observed level of divergence (~15% in *gag* and *pol* and ~30% in *env*) remains higher than that observed for the most distant isolates of HIV-1 (maximum ~10% in pol and ~20% in env[15]). Thus, classification of HIV-1, HIV-2 and SIV into either two or three subgroups of primate lentiviruses should remain an open issue at this time. Continued sequence analysis with additional authentic isolates will be needed to determine the extent to which the SIV and HIV-2 groups overlap and the genetic variability of this group of viruses. In any case, it already seems that the conserved regions defined by comparison of sequenced isolates of HIV-1 and HIV-2 (refs 12, 13) are also conserved in SIV (Fig. 3).

As already observed for different isolates of HIV-1 and HIV-2 (refs 12, 13), the transacting *tat* genes of HIV-2 and SIV show a large degree of divergence (40.8%). But preliminary experiments have shown that the clone λSIV1 encodes a functional transactivator with a similar specificity to that of HIV-2 *tat*—that is, it transactivates the HIV-2 LTR more efficiently than the HIV-1 LTR (ref. 12 and M. Emerman *et al.*, manuscript in preparation).

Theories regarding the possible existence of an animal reservoir for human AIDS and its role in the recent apparition of the AIDS pandemics must now take into account the sequence relationships of the lentiviruses and the fact that at least two divergent groups of lentiviruses are found in the human population. The observation that the divergence of all known lentiviruses (Table 1*b*) approximately follows the divergence of the infected species does not support a recent horizontal interspecies transmission. Sequence comparisons (Table 1*b*) reveal also that HIV-2 or African isolates of HIV-1, although obviously derived from a common ancestor, do not appear simply as evolutionary intermediates between SIV and European or US isolates of HIV-1. Thus, even if it is supposed that monkeys could be an accessory reservoir for HIV-2, available sequence data are inconsistent with the idea that AIDS emerged from recent transmission of SIV to humans, followed by rapid viral evolution toward HIV-1.

Recombinant DNA techniques have allowed efficient production of a variety of HIV-1 antigens (see for example refs 28–30) but adequate testing of such antigens as potential vaccines is limited by the absence of systems for live virus challenge. The availability of sequenced molecular clones should now permit the synthesis of similar products of SIV origin. These SIV antigens can be tested reliably in macaques, before testing analogous HIV antigens in humans.

We thank Drs F. Clavel, M. Emerman, L. Montagnier and S. Wain-Hobson for helpful discussions. The sequence data in this publication have been submitted to the EMBL/GenBank Libraries under the accession number Y00277.

Note added in proof: Following the submission of this paper, Hirsch *et al.* have reported a partial sequence of $STLV\text{-}3_{AGM}$ (*Cell* 49, 307–319; 1987). Their findings are similar to ours, in particular regarding the existence of an in-frame stop codon in the *env* gene at the same position as in our sequence. By comparison of the envelope proteins, $STLV\text{-}3_{AGM}$ appears to be closely related to SIV_{MAC} (91.4% of amino-acid identity).

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Characterization of Virus-Responsive Plasmacytoid Dendritic Cells in the Rhesus Macaque

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Plasmacytoid dendritic cells (PDC) are potent producers of alpha interferon (IFN-α) in response to enveloped viruses and provide a critical link between the innate and adaptive immune responses. Although the loss of peripheral blood PDC function and numbers has been linked to human immunodeficiency virus (HIV) progression in humans, a suitable animal model is needed to study the effects of immunodeficiency virus infection on PDC function. The rhesus macaque SIV model closely mimics human HIV infection, and recent studies have identified macaque PDC, potentially making the macaque a good model to study PDC regulation. In this study, we demonstrate that peripheral blood PDC from healthy macaques are both phenotypically and functionally similar to human PDC and that reagents used for human studies can be used to study macaque PDC. Both human and macaque PBMC expressed IFN-α in response to herpes simplex virus (HSV), the prototypical activator of PDC, as measured by using an IFN bioassay and IFN-α-specific enzyme-linked immunospot assays. Similar to human PDC, macaque PDC were identified by using flow cytometry as $CD123^+$ $HLA\text{-}DR^+$ $lineage^-$ cells. In addition, like human PDC, macaque PDC expressed intracellular IFN-α, tumor necrosis factor alpha, macrophage inflammatory protein 1β/CCL4, and IFN-inducible protein 10/CXCL10 upon stimulation with HSV, all as determined by intracellular flow cytometry. We found that IFN regulatory factor 7, which is required for the expression of IFN-α genes, was, similar to human PDC, expressed at high levels in macaque PDC compared to monocytes and $CD8^+$ T cells. These findings establish the phenotypic and functional similarity of human and macaque PDC and confirm the utility of tools developed for studying human PDC in this animal model.

Dendritic cells (DC) are ubiquitous cells found in blood, lymphoid, and many other nonlymphoid tissues. These heterogeneous cells share the ability to take up (11, 23, 36, 38, 46) and process and present (31) exogenous antigens to $CD4^+$ T cells (3, 5, 21, 44). Two distinct populations of DC have been identified in humans on the basis of their surface antigens: the myeloid DC (MDC), which are $lineage^-$, $CD11c^+$, $CD123^{dim}$, and $HLA\text{-}DR^+$ (32, 39), are phenotypically and functionally similar to monocyte-derived dendritic cells, which can be derived in vitro by culturing peripheral blood monocytes with granulocyte-macrophage colony-stimulating factor and interleukin-4 (37). These MDC produce little or no alpha interferon (IFN-α) in response to herpes simplex virus (HSV) (41). The second peripheral blood subset of DC, the plasmacytoid DC (PDC), are $lineage^-$, $CD11c^-$, $CD123^{bright}$, and $HLA\text{-}DR^+$ (32). Human PDC also express blood DC antigen 2 (BDCA-2) and BDCA-4, which are an endocytic C-type lectin receptor and neuropilin-1, respectively (10). PDC produce vast amounts of IFN-α (3 to 10 pg/cell or 1 to 2 IU/cell) in response to enveloped viruses such as HSV and Sendai virus (SV), in addition to some bacteria and DNA-containing unmethylated CpG sequences (4, 7, 15, 16, 25, 41). In addition, PDC have been shown to produce inflammatory chemokines such as macrophage inflammatory protein 1α (MIP-1α) and MIP-1β, IFN-inducible protein 10 (IP-10) and MCP-1 in response to CpG, inactivated influenza virus, CD40L stimulation, and HSV stimulation (28, 34), and HSV-stimulated PDC express chemokines that attract both natural killer (NK) cells and activated T cells (28). Depending on the nature of the stimulus they receive, PDC can direct either Th1 or Th2 responses (6, 26).

Our lab and others have shown that the functional and numerical loss of PDC in peripheral blood is associated with disease progression and enhanced virus replication in human immunodeficiency virus type 1 (HIV-1) (12, 13, 33, 42, 43)-, dengue virus (35)-, and HCV (1)-infected patients. The PDC therefore play a critical role in the link between innate and adaptive immunity, and understanding PDC function and their role in antiviral immunity is important for both vaccine design and therapeutic interventions.

Although murine models have been established for PDC, murine PDC are not phenotypically identical to human PDC, making direct correlations from mouse to human difficult (2, 19, 30). In addition, it has been challenging to study the progression of certain diseases, such as HIV infection, in humans. The difficulty in controlling for duration of infection, coinfection with other agents, and drug treatment and the difficulty in obtaining tissue samples from human patients all demonstrate

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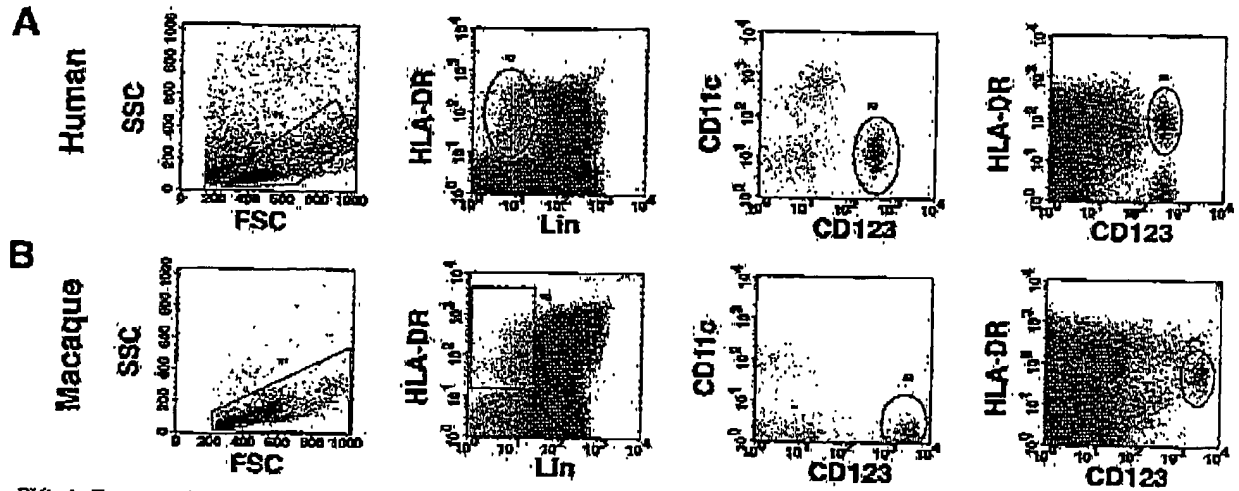

FIG. 1. Two-color flow cytometry gating identifies macaque PDC that phenotypically resemble human PDC. Human (A) and macaque (B) PDC were initially defined by using four-color flow cytometry. PBMC were first selected based on forward and side scatter (R1, left panels) and then further defined as HLA-DR$^+$ lineage$^-$ cells (R2, second panels) and CD123$^+$ CD11c$^-$ cells (R3, third panels). The same cells from the four-color flow cytometry, represented by the blue dots, are also identified by two colors as CD123$^+$ and HLA-DR$^+$ cells (R4, right panels). The data are representative of typical flow cytometric analysis.

the need for a model to study HIV infection. Because the immune system of the rhesus macaque closely resembles the human, the macaque model provides a unique system for studying PDC. Particularly because, as seen with progressive HIV infection in humans, the macaque shows an absolute decrease in CD4$^+$ T cells in SIV infection (17), the macaque provides an important animal model to study immunodeficiency virus pathogenesis.

Coates et al. have recently demonstrated that the PDC of fms-like tyrosine kinase 3-ligand (Flt3L)-treated rhesus macaques produce IFN-α in response to HSV, as demonstrated by enzyme-linked immunosorbent assay (8). The goal of the present study was to characterize PDC in the peripheral blood of healthy, untreated rhesus macaques. Our aim was to not only to establish whether rhesus PDC are phenotypically similar to human PDC but, more importantly, also to establish whether rhesus PDC are functionally similar to human PDC. We demonstrate here that macaque PDC, similar to human PDC, respond to live virus stimulation with IFN-α production. We show that many of the human reagents and techniques that we use to study human PDC in mixed preparations through intracellular flow cytometry, IFN bioassay, and enzyme-linked immunospot (ELISPOT) assay can also be applied to rhesus macaques. In addition, we demonstrate that macaque PDC, like their human counterparts (9, 22, 45), constitutively express high levels if IFN regulatory factor 7 (IRF-7) compared to monocytes and CD8$^+$ T cells. Finally, we demonstrate that, similar to humans (28), macaque PDC produce tumor necrosis factor alpha (TNF-α), IP-10/CXCL10, and MIP-1β/CCL4 in response to viral stimulation.

MATERIALS AND METHODS

Animals. Rhesus macaques (*Macaca mulatta*) were housed at the California National Primate Research Center in accordance with the regulations of the American Association for Accreditation of Laboratory Animal Care standards. All animals were negative for antibodies to HIV-2, simian immunodeficiency virus (SIV), type D retrovirus, and simian T-cell lymphotropic virus type 1.

Viruses. HSV type 1 (HSV-1) strain 2931 and vesicular stomatitis virus (originally obtained from Nicholas Ponzio, New Jersey Medical School) were grown, and titers were determined by plaque-forming assay in Vero cells (American Type Culture Collection, Manassas, Va.) as previously described (14). SV (Sendai/Cantell strain) was obtained from the Charles River SPAFAS, Inc. All virus stocks were stored at −70°C until use.

Cell lines. GM-0459A (GM; National Institute of General Medicine Sciences Human Genetic Mutant Cell Line Repository, Camden, N.J.), a primary fibroblast cell line trisomic for chromosome 21, was grown in Dulbecco modified Eagle medium (DMEM) (JHR Biosciences, Lenexa, Kans.) supplemented with 15% fetal calf serum (FCS; HyClone, Logan, Utah), 2 mM L-glutamine, 100 U of penicillin/ml, and 100 μg of streptomycin/ml (DMEM–15% FCS). Vero cells were grown in DMEM–10% FCS.

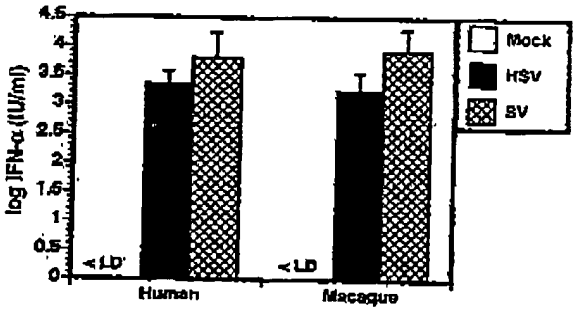

FIG. 2. Bioactive IFN produced by macaque and human IPC. Bioassays were performed to measure the IFN produced by PBMC incubated for 18 h with medium, HSV, or SV. For both macaques and humans, the IPC produced less than the lower limits of detection (LD) in response to mock (medium) stimulation. Mean values for six human samples and eight macaque samples are shown. The error bars represent one standard deviation from the means. There were no significant differences in IFN production between humans and macaques.

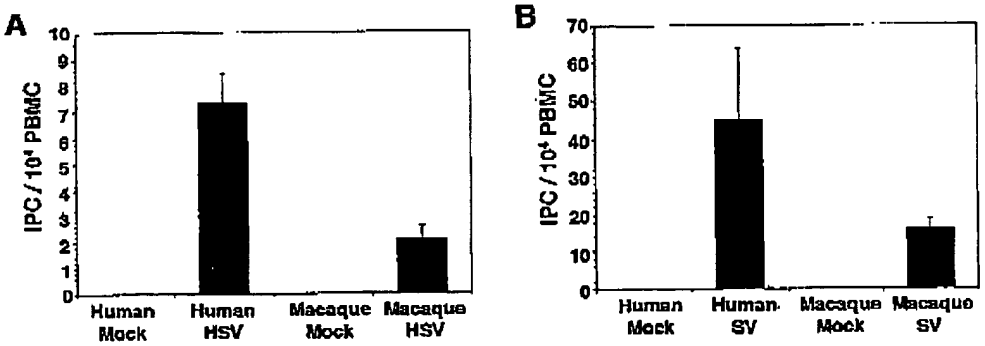

FIG. 3. Frequency of IFN-α-producing cells in macaque and human PBMC. Macaque and human PBMC were either mock stimulated (A and B), HSV stimulated (A), or SV stimulated (B) for 6 h. The frequency of IFN-α-producing cells was determined by ELISPOT assay. The mean values for 6 human samples and 12 macaque samples are shown. The error bars represent one standard error of the mean. The number of IPC detected in macaque PBMC in response to HSV and SV was significantly lower than in humans ($P < 0.05$).

Preparation of PBMC. Human blood was obtained with informed consent from healthy human donors. Rhesus blood was drawn into heparinized tubes and either tested fresh at the University of California at Davis or shipped at room temperature overnight from the California National Primate Research Center to New Jersey. Human and macaque peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque density centrifugation (Lymphoprep; Accurate Chemical and Scientific Co., Westbury, N.Y.). PBMC were washed twice with Hanks balanced salt solution (Life Technologies, Grand Island, N.Y.) and resuspended in RPMI 1640 (Life Technologies) containing 10% FCS, 2 mM L-glutamine, 100 U of penicillin/ml, 100 μg of streptomycin/ml, and 25 mM HEPES and then enumerated electronically with a Series Z1 Coulter Counter (Coulter Electronics, Inc., Hialeah, Fla.).

For some experiments, freshly isolated macaque PDC were immediately frozen in 10% dimethyl sulfoxide (Sigma-Aldrich, St. Louis, Mo.)–90% fetal bovine serum, stored in liquid nitrogen, shipped from California to New Jersey on dry ice, and then stored again in liquid nitrogen until thawing.

Flow cytometry. For surface staining, cells were washed with cold 0.1% bovine serum albumin (BSA; Sigma-Aldrich) in phosphate-buffered saline (PBS) (Life Technologies), blocked with 5% heat-inactivated human serum, stained with fluorochrome-conjugated antibody for 20 min at 4°C, washed, and fixed with 300 μl of 1% paraformaldehyde in PBS (Fisher, Pittsburgh, Pa.) at 4°C overnight. The antibodies used for surface staining were as follows: CD8 (clone SK1), CD14 (clone7G3), CD123 (clone MφP9), and HLA-DR (clone L243) (BD Biosciences, San Diego, Calif.).

Intracellular detection of IFN-α, TNF-α, IRF-7, and chemokines. PBMC were prepared for intracellular detection of IFN-α, IRF-7, CXCL10/IP-10, and CCL4/MIP-1β by using a modification of the method described previously (29). PBMC (2×10^6 cells/ml)were either mock stimulated (placed in the incubator without any virus added) or stimulated with HSV-1 strain 2931 at a multiplicity of infection of 1 for 4 h at 37°C in 5% CO_2. Brefeldin A (5 μg/ml) (Sigma-Aldrich) was then added, and incubation was continued for an additional 2 h. Cells were surface stained, as described above, and fixed with 1% paraformaldehyde in PBS at 4°C overnight. The following day, cells were washed twice with PBS–2% FCS, permeabilized with 0.5% saponin (Sigma-Aldrich) in PBS–2% FCS for 30 min at room temperature, and then incubated with 50 ng of biotinylated 293 monoclonal antibody (MAb) to IFN-α (obtained from G. V. Alm, Uppsala, Sweden) or a commercially available antibody to IFN-α (clone MMHA-2, PBL Biomedical Laboratories, Piscataway, N.J.). Biotinylation was carried out by using the succinamide ester method. For intracellular staining of IRF-7, chemokines, and TNF-α, polyclonal rabbit antibody to IRF-7 (Santa Cruz Biotechnology, Inc., Santa Cruz, Calif.), anti-CCL4 (R&D Systems, Minneapolis, Minn.), or biotinylated anti-CXCL10 (U.S. Biologicals, Swampscott, Mass.), or anti-TNF-α (BD Pharmingen) were incubated with the PBMC for 30 min at room temperature. Cells were subsequently washed twice with 0.5% saponin in PBS–2% FCS and incubated 30 min at room temperature with streptavidin-Quantum Red (Sigma-Aldrich) or fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin G (IgG; BD Biosciences). Finally, the cells were washed and resuspended in 1% paraformaldehyde in PBS and analyzed by using a FACSCalibur flow cytometer with CellQuest analysis software (BD Biosciences).

ELISPOT assays. ELISPOT assays for detection of IFN-α-producing cells were carried out as previously described (13). Briefly, PBMC (10^6/ml) were either mock stimulated (placed in the incubator with no virus) or stimulated for 6 h with HSV-1 at an multiplicity of infection of 1. We coated 96-well Multiscreen plates (Millipore) with AS94 (Glaxo SmithKline, Uxbridge, United Kingdom), a bovine polyclonal antibody to IFN-α, for 5 h. Cell suspensions were added, and this was followed by incubation for 11 h at 37°C; the primary antibody to IFN-α, MAb 293, was then added. After a 2-h incubation at room temperature, secondary antibody, horseradish peroxidase-conjugated goat anti-mouse IgG (Jackson Immunoresearch Laboratories), was added for 1 h. Finally, diaminobenzidene with H_2O_2 was added for 5 min. After washing and drying steps, the spots were counted under a dissecting microscope to determine the number of IFN-producing cells (IPC).

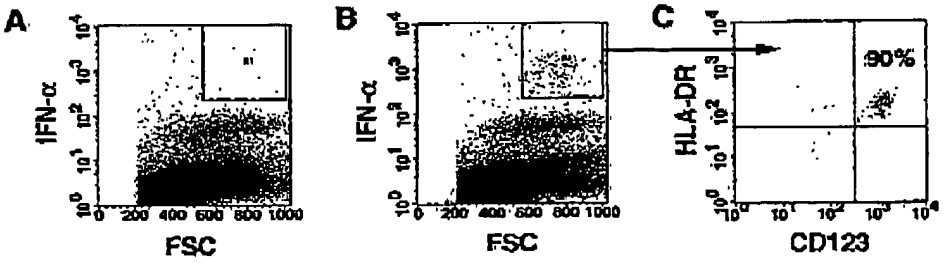

FIG. 4. PDC are the predominant IPC in HSV-stimulated macaque PBMC. PBMC were either mock (A) or HSV (B) stimulated and gated based on forward scatter and intracellular expression of IFN-α. (C) Of the cells that are IFN-α$^+$, 90% are HLA-DR$^+$ and CD123$^+$. The data are shown for one representative experiment of two.

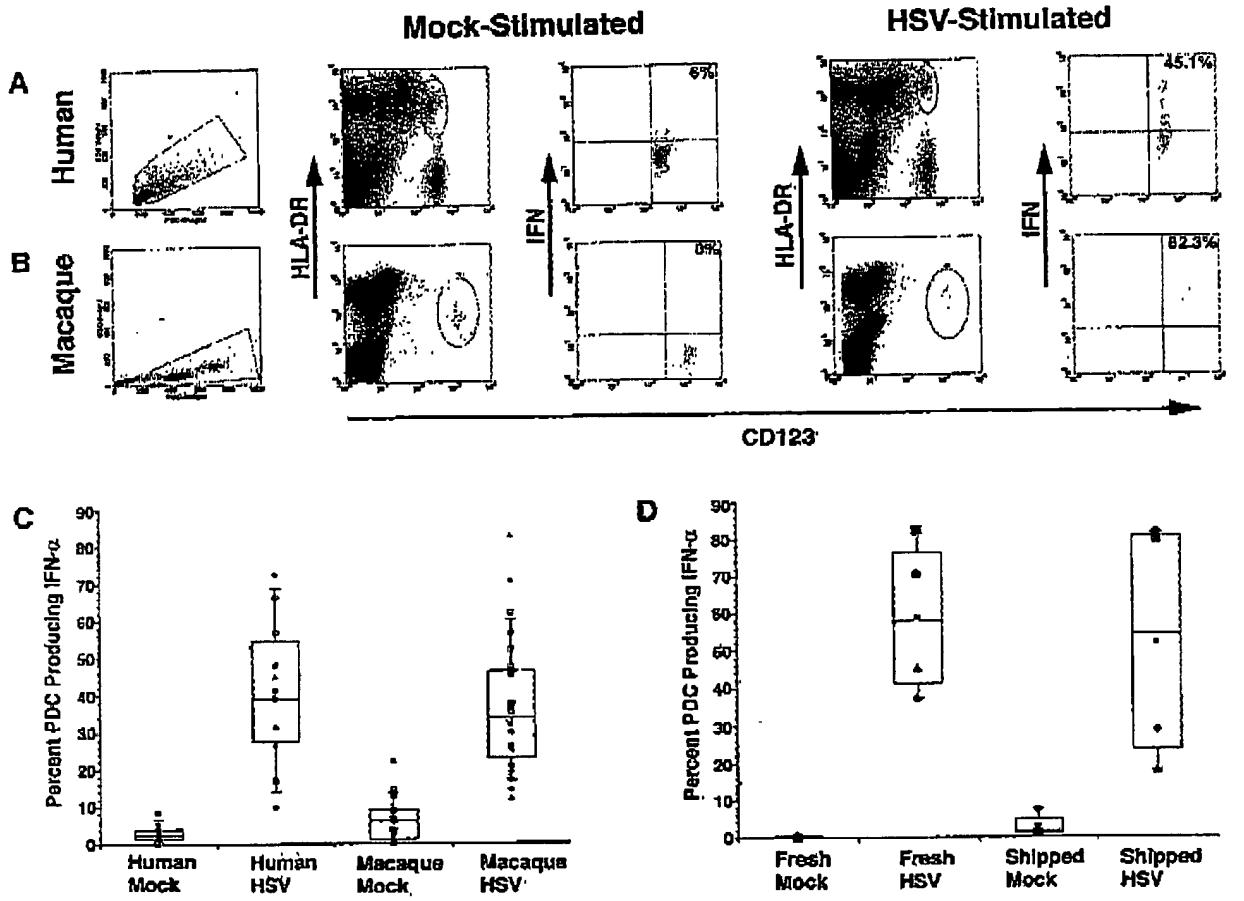

FIG. 5. Intracellular flow cytometric detection of IFN-α production by HSV-stimulated macaque and human PDC. (A) Human PBMC (left panel) were gated as $CD123^{+}$ HLA-DR^{+} cells and then further gated for IFN-α production to determine the percentage of PDC that produce IFN-α in response to mock and HSV stimulation. The numbers in the upper right quadrants are the percentages of IFN-α^{+} PDC. (B) Macaque PBMC were gated as described for human PDC in panel A to determine the percentage of PDC producing IFN-α in response to mock and HSV stimulation. The data shown are representative experiments of 11 human samples and 28 macaque samples. (C) The percentage of PDC producing IFN-α ranged from 12 to 80% in humans and 36 to 82% in macaques. (D) There was no significant difference in the percentages of PDC producing IFN-α between freshly drawn macaque blood and overnight-shipped blood, where PBMC derived from fresh and shipped blood are shown with the same symbol for an individual animal. The boxes in panels C and D represent the upper and lower quartiles, with the medians shown inside the boxes. The lines extend out to either the upper quartiles plus 1.5 times the interquartile range or the lower quartiles minus 1.5 times the interquartile range.

IFN bioassays. IFN bioassays were performed by using a cytopathic effect reduction assay with GM cells infected with vesicular stomatitis virus as the challenging virus as previously described (14). An IFN-α reference standard (G-023-901-527; National Institute of Allergy and Infectious Disease, Bethesda, Md.) was used at 100 IU/ml.

Purification of PDC from macaque blood. PBMC were depleted of T cells, B cells, NK cells, and monocytes by using a modified version of the magnetic blood dendritic cell isolation kit (Miltenyi Biotech, Inc., Auburn, Calif.). Briefly, PBMC were washed and resuspended in MACS buffer (PBS [Life Technologies] with 0.5% BSA and 2 mM EDTA [Sigma-Aldrich]) and then incubated at 4°C with anti-CD3, anti-CD16 beads, anti-CD14 beads, anti-IgG1 beads, and anti-CD20 beads. PDC were negatively selected for by using the MACS magnetic separation column (LD columns).

PDC were subsequently positively selected for by incubating the negatively selected cells at 4°C for 20 min with anti-CD123 PE. Resuspended cells were then incubated with anti-phycoerythrin beads (Miltenyi Biotech) for 15 min, and PDC were positively selected by using a MACS magnetic separation column (LS and MS columns).

Giemsa staining. PDC were enriched by using the magnetic bead separation method described above, cytospin centrifuged onto slides, and allowed to air dry overnight. PDC were stained with Giemsa stain, mounted with Permount (Fisher), and observed under a microscope.

Identification of IFN-α-producing PDC by fluorescence microscopy. PDC were enriched by using the negative selection described above. Enriched PDC were either mock or HSV stimulated for 6 h, after which they were positively selected, as described above. The resulting cells were then subjected to cytospin centrifugation and allowed to air dry on slides overnight. Purified PDC were fixed with 1% paraformaldehyde in PBS for 15 min and then permeabilized with 0.2% Triton X-100 in PBS for 5 min. Slides were subsequently washed twice with PBS and blocked with 3% BSA in PBS and 10% normal goat serum for 30 min. Purified PDC were incubated for 30 min with mouse MAb to human IFN-α (clone MMHA-2; PBL) that was labeled with Alexa Fluor-680 by using the Zenon labeling system (Molecular Probes, Eugene, Oreg.). Cells were washed twice with 0.2% Triton X-100 in PBS and washed twice with PBS. Purified PDC were mounted on slides with mounting medium (Vector) and then observed under a fluorescence microscope.

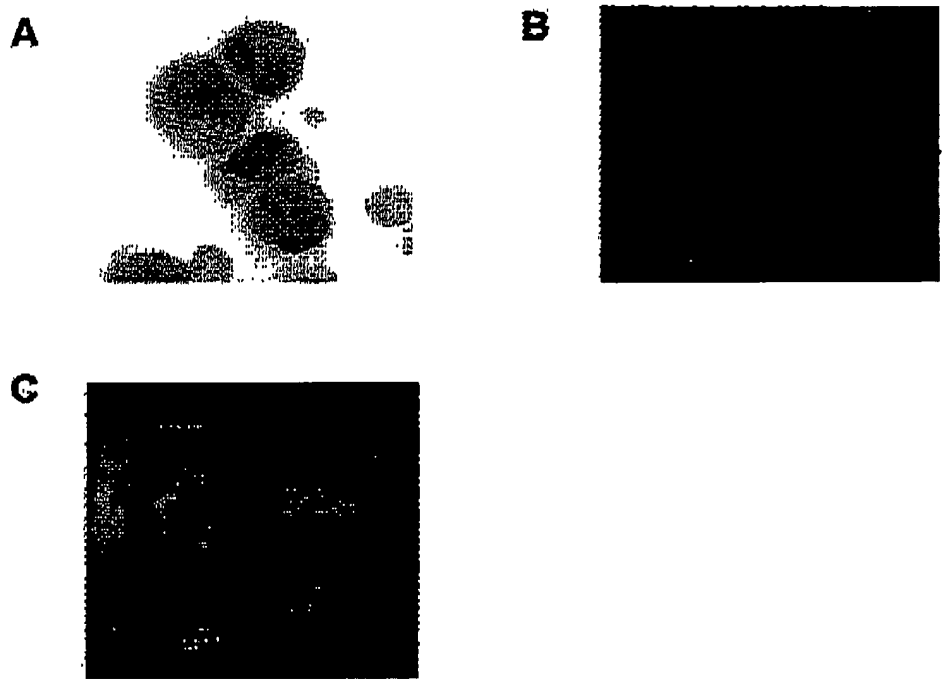

FIG. 6. Microscopic analysis of macaque PDC. (A) Macaque PDC were enriched from PBMC by negative selection and then Giemsa stained and analyzed for morphology, revealing cells with characteristic PDC morphology. Positively selected PDC were mock (B) or HSV (C) stimulated for 6 h and then stained with Alexa Fluor 680-conjugated anti-IFN-α antibody.

Statistical analysis. Data are expressed as mean values plus standard deviations. Statistical significance was determined by one-way analysis of variance with Scheffe's test. Differences were considered to be significant at P values of <0.05.

RESULTS

Phenotypic characterization of macaque PDC. Human PDC constitute <1% of PBMC, making them difficult to isolate and study in large numbers. However, human PDC can be identified by using flow cytometry, allowing us to gate in on the small population of cells for both phenotypic and functional analysis (13). Human PDC have been characterized as cells that are lineage marker negative, HLA-DR$^+$, CD123^{bright}, and CD11c$^-$ (Fig. 1A). Moreover, we have shown that the cells identified as HLA-DR$^+$ and CD123^{bright} by four-color fluorescence-activated cell sorting (FACS) analysis are identical to the HLA-DR$^+$ and CD123^{bright} population by using a simplified two-color analysis (Fig. 1A) (9). In addition, BDCA-2 and BDCA-4 have been used to identify human PDC in PBMC populations (8).

Using the same four- and two-color FACS analyses used to identify human PDC, we identified a population of cells in rhesus blood that is HLA-DR$^+$ CD123^{bright} and thus phenotypically resemble human PDC (Fig. 1B). Interestingly, there was more variability in the mean fluorescence intensity (MFI) of expression of HLA-DR in macaque PDC than in the human PDC. However, antibodies to human PDC-specific surface markers BDCA-2 and -4 did not cross-react with macaque PDC (data not shown). Gating on the HLA-DR$^+$ CD123^{bright} cells, there were, on average, 1,260 ± 411 PDC/3 × 10^5 PBMC for humans (0.4%, n = 11 donors) and 264 ± 189 PDC/3 × 10^5 PBMC for macaques (0.1%, n = 28 donors) (P < 0.05). Phenotypic analysis comparing PDC from matched donors in freshly isolated PBMC, in PBMC isolated in shipped peripheral blood, and in frozen PBMC yielded similar results (data not shown).

HSV-induced IFN-α production by rhesus PBMC. In humans, the total IFN response can be tested by measuring IFN-α release (as determined by IFN bioassay) after in vitro stimulation of PBMC with HSV (12). To determine whether this assay can also be used to assess IFN-α production in rhesus PBMC, these cells were stimulated in vitro with HSV for 18 h, and the IFN-α in supernatants was measured. Positive control cultures consisted of supernatants from human PBMC stimulated with HSV.

In both human and macaque cultures, unstimulated PBMC produced less IFN than the lower limits of detection of the assay (Fig. 2). In response to HSV stimulation, human PBMC produced a geometric mean of 2,220 IU of IFN/10^6 cells (one standard deviation; range, 851 to 5,791), whereas macaque PBMC produced a geometric mean of 1,723 IU of IFN/10^6 cells (one standard deviation; range, 867 to 3,424) (P = 0.66 [not significant]). In addition, in response to SV, which stimulates both monocytes and human PDC to produce IFN-α (13), human PBMC produced a geometric mean of 6,049 IU of IFN/10^6 cells (one standard deviation; range, 2,147 to 17,044), whereas macaque PBMC produced a geometric mean of 7,965

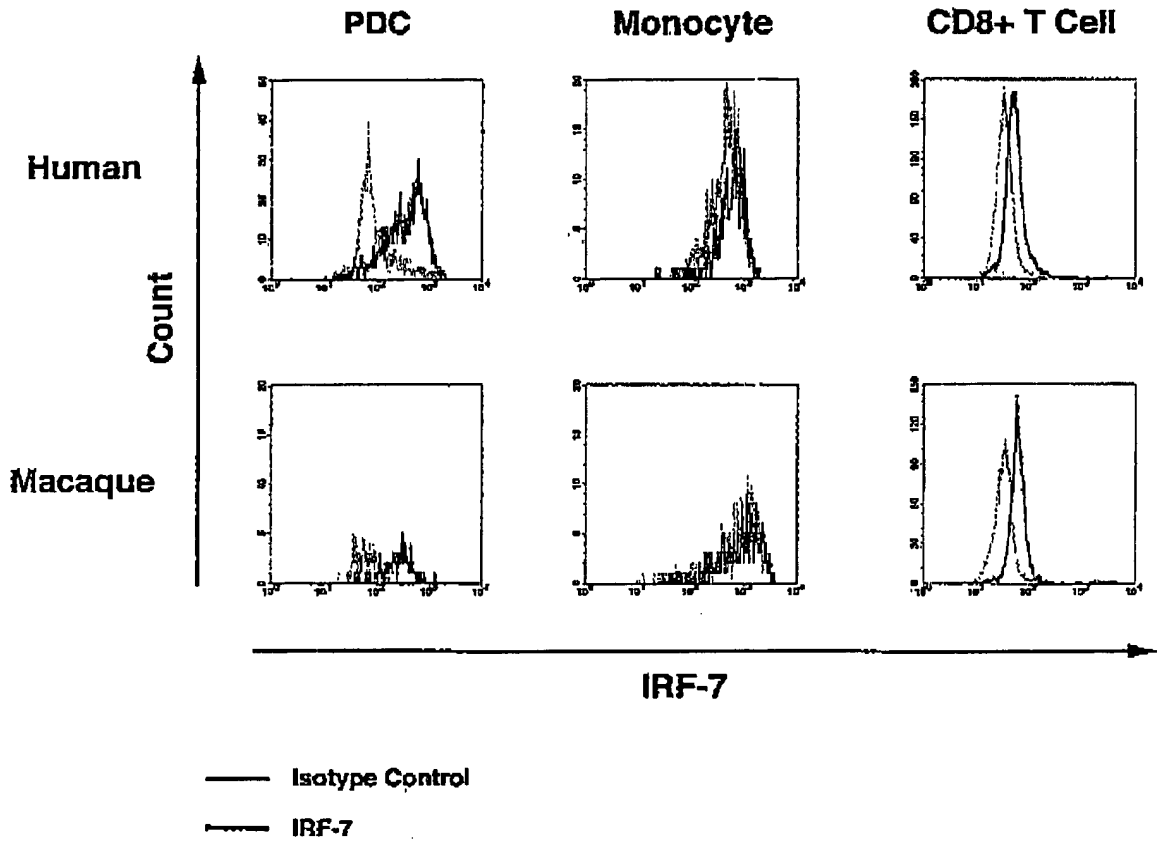

FIG. 7. Expression of IRF-7 in human and macaque PDC, monocytes, and $CD8^+$ T cells. PBMC from humans (upper panels) and macaques (lower panels) were surface labeled for identification of PDC, monocytes, and $CD8^+$ T cells. Cells were then permeabilized and stained with either control antiserum (dim lines) or anti-IRF-7 (bold lines), followed by fluorescein isothiocyanate-conjugated goat anti-rabbit IgG. PDC ($CD123^+$ $HLA\text{-}DR^+$), monocytes ($CD14^+$), and $CD8^+$ T cells were gated, and intracellular expression of IRF-7 in the selected populations was determined as for the PDC. The data are representative of two experiments with similar results.

IU of IFN/10^6 cells (one standard deviation; range, 3,250 to 19,518) ($P = 0.62$, NS). Thus, rhesus PBMC responded to HSV stimulation with a magnitude of IFN-α secretion similar to that of human PBMC.

To further determine whether the macaque model closely resembles the established human model, the frequency of HSV-responsive IPC was determined by using an IFN-α-specific ELISPOT assay. HSV and SV stimulation of human PBMC yielded average frequencies of 7.3 ± 2.7 and 45.1 ± 46.8 IPC/10^4 PBMC, respectively, whereas averages of 2.1 ± 1.8 and 15.8 ± 9.4 IPC/10^4 PBMC, respectively, were detected in rhesus samples after HSV and SV stimulation (Fig. 3). Both the HSV-induced ($P = 0.0002$) and SV-induced ($P = 0.0473$) ELISPOT frequencies were significantly lower in macaques than in humans. Moreover, in general, the sizes of the "spots" in the ELISPOT assays, as determined by visual observation, were smaller in macaque samples than in human samples.

Identification of PDC as the major IFN-α-producing cells after in vitro stimulation of rhesus PBMC with HSV. To determine whether rhesus PDC, like their human counterparts, are indeed the main IFN-α-producing cell type, PBMC from both macaques and humans were stimulated with HSV-1 for 6 h and then stained and analyzed by FACS for intracellular IFN-α. As in humans, the majority of the cells staining positive for IFN-α were $CD123^+$ cells (Fig. 4). Using the same gating strategy as described above for enumeration of PDC, the percentages of PDC producing IFN-α in response to 6 h of stimulation with HSV were determined for humans and macaques (representative results are shown in Fig. 5A and B, respectively). As we previously reported for human PDC (12), not all of the macaque PDC produced IFN-α simultaneously upon viral stimulation. The percentages of PDC producing IFN-α varied from subject to subject (Fig. 5C), ranging from 10 to 72% in human PDC and from 14 to 82% in macaque PDC. Although we only directly compared a limited number of fresh versus shipped blood samples for expression of IFN-α after HSV stimulation, there was no statistical difference between these data (Fig. 5D). In contrast, although PDC in cryopreserved PBMC samples were similar in terms of phenotype and frequency to fresh PDC, there was variability in their ability to

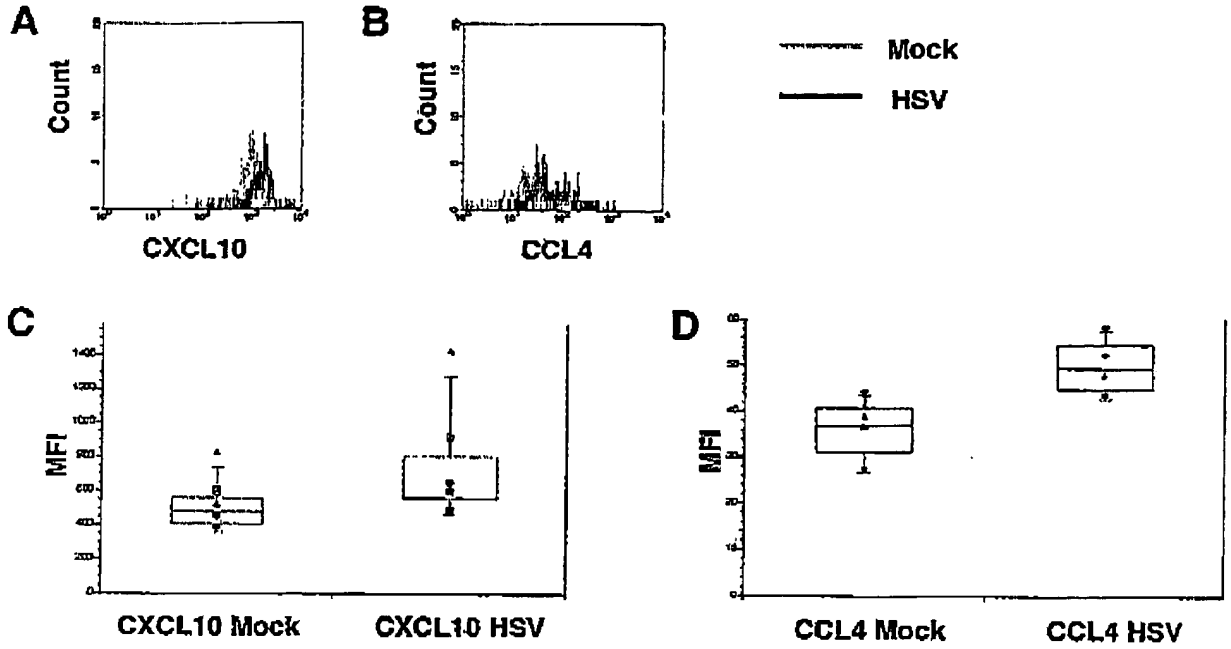

FIG. 8. CXCL10 and CCL4 production in macaque PDC. Macaque PBMC were either mock or HSV stimulated for 6 h. PDC were gated as $CD123^+$ $HLA\text{-}DR^-$ cells as described above. The intracellular expression of CXCL10 (A) and CCL4 (B) was measured by flow cytometry. CXCL10 and CCL4 production is compared between mock stimulation (dim lines) and HSV stimulation for 6 h (bold lines). Panels A and B are representative experiments of seven samples that showed an increase in CXCL10 in response to HSV and four samples for CCL4. The differences in MFI for CXCL10 (C) and CCL4 (D) are shown. Boxes are as described for Fig. 5. There was a significant difference in expression of both CXCL10 and CCL4 between mock and HSV stimulation.

produce IFN-α. Similar variability was obtained with cryopreserved human PBMC samples, indicating that our method of freezing yielded samples with inconsistent functional ability (data not shown).

Morphology of macaque PDC. PDC were enriched from PBMC by negative selection and subsequently Giemsa stained (Fig. 6A), revealing enrichment for large cells with lateralized reniform nuclei, a typical PDC morphology. For fluorescence microscopy, negatively enriched PDC were further purified by positive selection, stimulated with HSV for 6 h, and stained with anti-IFN-α. Virtually no IFN-α positive cells were seen in the mock-stimulated purified PDC (Fig. 6B), whereas the HSV-stimulated, purified PDC showed a bright fluorescence pattern in the cytoplasm of the cells (Fig. 6C).

IRF-7 expression in rhesus PDC. IRFs play an important role in the induction of IFN-α and IFN-β gene expression, with IRF-7 being specifically required for stimulation of the IFN-α genes (27, 40, 47). We (9, 22) and others (45) have previously reported that IRF-7 is expressed at high constitutive levels in human PDC and at much lower levels in monocytes and T cells, thus making the PDC uniquely poised to rapidly produce high levels of IFN-α in response to virus stimulation. Similar to human PDC, constitutive high levels of IRF-7 expression were observed in macaque PDC, with lower levels being observed in monocytes and $CD8^+$ T cells (Fig. 7). Macaque PDC, however, had lower MFIs associated with IRF-7 than did human PDC (i.e., MFI = 127.9 [one standard deviation range from 85.6 to 191.0] versus MFI = 376.7 [one standard deviation range from 181.0 to 786.2], respectively; $P = 0.0003$) (9, 22).

Macaque PDC produce CXCL10/IP-10, CCL4/MIP-1β, and TNF-α in response to HSV stimulation. IP-10 (CXCL10) and MIP-1β (CCL4) are inflammatory chemokines that chemoattract Th1-polarized T cells and NK cells, respectively. We have previous demonstrated that human PDC produce CXCL10 in response to either IFN-α or HSV, whereas HSV but not IFN-α induces the expression of CCL4 (28). In the macaque model, utilizing intracellular flow cytometry, we detected significant IP-10/CXCL10 production in HSV-stimulated PDC from 7 of 11 monkeys tested (Fig. 8A and C). We also observed HSV-induced upregulation of MIP-1β/CCL4 expression in macaque PDC (Fig. 8B and D), a finding similar to what we reported earlier for humans (28). Finally, similar to human PDC, macaque PDC expressed intracellular TNF-α in response to HSV stimulation (Fig. 9).

DISCUSSION

Although it is recognized that PDC play a critical role in the link between innate and adaptive immunity and that their numerical and functional dysfunction contributes to HIV pathogenesis (12, 42), study of the fate of PDC in HIV infection has been hampered by the difficulty of monitoring the PDC throughout the body. Likewise, it is difficult to study PDC in human hosts at the earliest periods after infection with HIV.

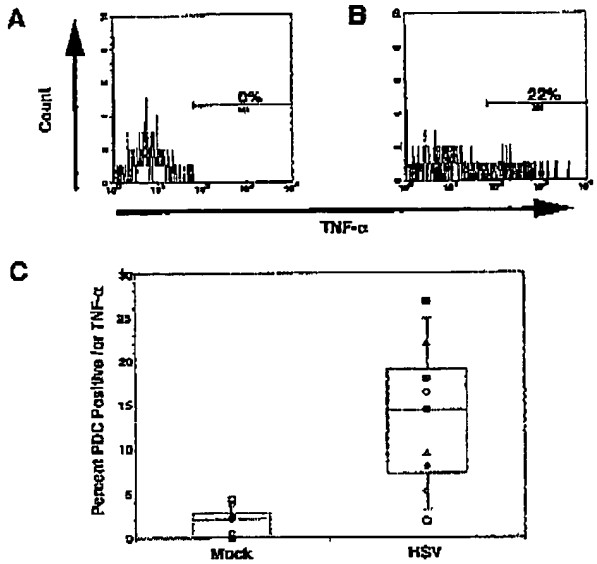

FIG. 9. TNF-α expression in macaque PDC. Macaque PBMC were either mock or HSV stimulated for 6 h and then surface stained for identification of PDC, followed by permeabilization and staining for TNF-α expression. The percentages of PDC that were positive for TNF-α are shown for mock (A)- and HSV (B)-stimulated PBMC for one representative monkey out of nine. (C) The percentage of PDC positive for TNF-α ranged from 0 to 4% in mock-stimulated PBMC and 2 to 27% in HSV-stimulated PBMC ($P < 0.05$).

Thus, an animal that allows study of PDC function in the context of immunodeficiency virus infection is very much needed. The present study was undertaken to determine the extent to which macaque PDC are similar to their human counterparts.

We used reagents that we routinely use in the study of human PDC to further describe the rhesus macaque PDC. Others have reported that macaque PDC, like their human counterparts, can be identified by using four markers: lineage, HLA-DR, CD123, and CD11c (8, 48). We demonstrate here that the macaque PDC, like their human counterparts, can be identified by using our two-color scheme (9), which utilizes CD123 and HLA-DR only. Using a four-color flow cytometer, defining the PDC by two colors, opens up two additional channels for additional studies, such as intracellular analysis of IFN-α, chemokines, or IRF-7. Two existing antibodies frequently used to identify and/or isolate human PDC, namely, BDCA-2 and BDCA-4, however, failed to react with the macaque PDC PDC phenotype, and frequencies were found to be similar within macaque PBMC obtained from freshly isolated blood, PBMC separated from heparanized blood that had been shipped overnight, and in cryopreserved, thawed PBMC. The macaque, however, had a significantly lower percentage of PDC in the peripheral blood than human donors. In addition, we observed more variability in the expression of HLA-DR by the macaque than the human PDC, but this did not interfere with our ability to identify the PDC. By using Giemsa stain, isolated PDC were indistinguishable from human PDC.

In addition to their phenotypic similarity to human PDC, the macaque PDC within the PBMC vigorously produced IFN-α in response to stimulation with HSV, as measured both by total IFN-α activity in an IFN bioassay and by ELISPOT analysis with human IFN-α specific reagents. Although the levels of IFN in supernatants of HSV and SV-stimulated samples were statistically indistinguishable, the ELISPOT frequencies of the IPC were lower in macaques than in humans. The lower frequency of HSV-responsive IPC, as measured by ELISPOT, is consistent with the observation that the monkeys had a lower percentage of PDC among PBMC than humans. The ability of the gated PDC to produce IFN-α, as measured as the percent PDC positive for intracellular IFN-α, was statistically equivalent between monkeys and humans, indicating that, as we previously demonstrated in humans (12, 28), not all PDC respond to HSV with IFN production, a finding that has also been seen with human PDC stimulated with the TLR7 agonist, imiquimod (18). The markedly lower frequency of SV-responsive IPC in monkeys compared to humans may reflect limitations to the ELISPOT assay. SV is known to induce both PDC and monocytes to produce IFN-α, with the monocytes expressing 5- to 10-fold lower expression of IFN-α on a per-cell basis than the PDC (13, 20). In the ELISPOT, this is seen by a mixture of small (monocyte-derived) and large (PDC-derived) spots. The number of smaller, monocyte-derived IFN-α spots was noticeably lower in the macaque than in the human, perhaps reflecting spots that were too dim to detect, thus limiting the usefulness of the ELISPOT assay for detecting SV-induced IPC.

Also similar to the human PDC, macaque PDC produced both CXCL10/IP-10 and CCL4/MIP-1β, as well as TNF-α, in response to HSV. Thus, as in humans, the macaque PDC are uniquely poised to interact with other cell types such as NK cells and T cells (28) and to link innate and adaptive immune responses (24). Overall, the similarity of macaque PDC to human PDC in response to HSV demonstrates the usefulness of the macaque model for the study of PDC.

Coates et al. studied PDC in Flt3L-treated macaques (8). Although growth factors such as Flt3L may be useful in therapeutics, we have demonstrated that fresh, untreated PDC can be functionally studied in the macaque model. In addition, we were able, by using the two-color scheme to identify PDC, to demonstrate that macaque PDC, like their human counterparts, produce IFN-α, IP-10, MIP-1β, and TNF-α in response to viral stimulation. The similarity in cytokine production of macaque to human PDC further establishes the macaque model as a good system for studying PDC.

In addition to the phenotypic and functional similarities between the macaque and human PDC, the macaque PDC, again similar to human PDC (9, 22), were found to express high levels of the transcription factor IRF-7 compared to other peripheral blood cell types. In humans, we have demonstrated that this IRF-7 can be rapidly translocated to the nucleus of PDC after stimulation with HSV. We postulate that this high constitutive IRF-7 is what makes PDC such exquisite "professional IFN-producing cells" (40).

In conclusion, the macaque PDC model provides a valuable system to study these important cells in a nonhuman primate setting. Furthermore, the similarity between SIV and HIV pathogenesis in rhesus macaques and humans, respectively, provides a useful model in the macaque for studying HIV

pathogenesis. Studies are currently under way to evaluate the PDC system in the context of acute and chronic immunodeficiency virus infection. The demonstration of the macaque as a good model for PDC study will hopefully permit the elucidation of the role of PDC in viral pathogenesis, as well as in other human diseases.

ACKNOWLEDGMENTS

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Review

Immunity to retroviral infection: The Friend virus model

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ABSTRACT **Friend virus infection of adult immunocompetent mice is a well established model for studying genetic resistance to infection by an immunosuppressive retrovirus. This paper reviews both the genetics of immune resistance and the types of immune responses required for recovery from infection. Specific major histocompatibility complex (MHC) class I and II alleles are necessary for recovery, as is a non-MHC gene, Rfv-3, which controls virus-specific antibody responses. In concordance with these genetic requirements are immunological requirements for cytotoxic T lymphocyte, T helper, and antibody responses, each of which provides essential nonoverlapping functions. The complexity of responses necessary for recovery from Friend virus infection has implications for both immunotherapies and vaccines. For example, it is shown that successful passive antibody therapy is dependent on MHC type because of the requirement for T cell responses. For vaccines, successful immunization requires priming of both T cell and B cell responses. *In vivo* depletion experiments demonstrate different requirements for CD8* T cells depending on the vaccine used. The implications of these studies for human retroviral diseases are discussed.**

Scientific knowledge of retroviral infections in humans is relatively new and little is known about the types of immune responses required to successfully defend against these infections. Such knowledge would be extremely valuable for designing vaccines and immunomodulatory therapeutics. Studies of long term survivors of HIV infection are beginning to provide some insights (1–6), but such individuals are rare, and data are difficult to obtain. In general, cell-mediated responses rather than antibodies are considered the critical elements responsible for resolving most human viral infections. This is because humans with genetic deficiencies in T lymphocytes are very susceptible to many viral infections whereas those with antibody deficiencies are not (7, 8). However, antibody responses also appear essential for resistance against certain viruses such as enterovirus (9) and rabies virus (10), and there are numerous examples of antibodies curing or preventing viral infections (11–17). Thus, there remains controversy regarding which arms of the specific immune system are most important for resolving viral infections. Most likely this resolution depends on the specific virus and host involved, and often more than one aspect of the immune response is important, if not essential.

This review summarizes studies from the polycythemia-inducing strain of Friend virus (FV) complex, an immunosuppressive retrovirus model that induces leukemia in mice. The results indicate that resolution of retroviral infections may require more complex immunological responses than have been found for most other viruses. Numerous experiments using both genetic and immunological approaches demonstrate that immune resistance to FV requires multiple arms of the immune system, including $CD4^+$ T cells, $CD8^+$ T cells, and B cells, each providing essential nonoverlapping functions.

When adult mice of susceptible strains are infected with FV, their spleens rapidly enlarge because of virus-induced polyclonal proliferation of erythroid precursor cells (19–21). Subsequent proviral integration at the Spi-1 (*ets*) oncogene locus (22–27) combined with inactivation or mutation of the p53 tumor suppressor gene (28–30) produces fully malignant erythroleukemias. This process results in gross splenomegaly at 8–9 days postinfection and transplantable erythroleukemia cells as early as 15–20 days postinfection (31). Thus, a successful immune response must develop quickly enough to keep ahead of this transformation process.

Genes Involved in Recovery from FV Leukemia. Mice have evolved a formidable array of genes involved in conferring immunological resistance to FV-induced disease, including at least four major histocompatibility complex (MHC) (H-2) genes (32–35) and one non-MHC gene, Rfv-3 (36). In addition, there are six genes (Fv-1–Fv-6) that confer resistance to infection through nonimmunological mechanisms (37–39). Adult mice with appropriate susceptibility alleles at the nonimmunological loci are infectable by FV and develop severe splenomegaly. Their subsequent survival is dependent on MHC and Rfv-3 genes that control immunological responsiveness. Mice having high recovery MHC and Rfv-3 genotypes, such as $H\text{-}2^{b/b}$ and $Rfv\text{-}3^{r/s}$, spontaneously recover to near normal spleen size within several weeks and generally live out a normal life-span. Occasionally mice may eventually relapse, indicating the presence of persistent infection (40), but this aspect will not be further discussed. Experiments with MHC recombinant mice show that MHC regions H-2A, E, D, and T are important for recovery from acute FV infection.

The H-2D region of the mouse MHC has a very potent influence on recovery from FV infection because it encodes the class I molecules that present viral antigens to CTL (39). Of interest, the H-2D region also influences the kinetics of virus-specific $CD4^+$ helper T cell responsiveness (41) and controls host susceptibility to FV-induced immunosuppression (ref. 42; Table 1). The H-2D region exhibits an unusual gene–dose effect whereby $H\text{-}2D^{h/h}$ mice show the highest recovery incidence, $H\text{-}2D^{b/d}$ mice are intermediate, and $H\text{-}2D^{d/d}$ mice are lowest. Each of these genotypes differs in various FV-specific immune parameters (Table 1). One obvious way such a gene–dose effect might occur is through altering expression levels of the D^b class I molecules used to present viral peptides to cytotoxic T lymphocyte (CTL). However, experiments to test this hypothesis in the FV system indicate that D^b-associated high recovery did not require homozygous levels of D^b expression (43). An alternative that also has been investigated is whether expression of low recovery alleles, such as D^d, might produce a negative influence on

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Abbreviations: FV, Friend virus; MHC, major histocompatibility complex; CTL, cytotoxic T lymphocyte; F-MuLV, Friend murine leukemia virus; NK cell, natural killer cell

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Table 1. Gene dosage effects of H-2D genotype

H-2D genotype	FV-specific T cell responses		Recovery from FV*		
	CD4+ T cell proliferation†	CD8+ CTL‡	Low FV dose	High FV dose	FV-induced immunosuppression§
b/b	rapid	+++	yes	yes	no
b/d	slow	++	yes	no	no
d/d	negative	+	no	no	yes

*Low dose, 100 spleen focus forming units; high dose, 1000 spleen focus forming units.
†Kinetics of FV-specific CD4+ T cell proliferative responses after challenge with high dose of FV (rapid, 6 days; slow, 16 days).
‡The magnitude of FV-specific CTL responses is influenced by both the H-2D type and by the FV dose used for infection (41).
§Significant decrease in antibody response to sheep red blood cell challenge.

recovery. For instance, D^d gene products could delete potential Friend-specific T cells by negative selection during development in the thymus. Experiments with D^d transgenic mice showed that expression of D^d in an H-2^b mouse did not adversely impact recovery (44). It is also possible that some of the effects associated with H-2D are mediated by other genes that are very closely linked to H-2D and have not been separated from H-2D in the MHC recombinants used for mapping experiments. Possibilities include the tumor necrosis factor complex and the H-2L gene.

Two class II MHC genes, H-2A and H-2E, also play important roles in immunity to FV. For H-2A, high recovery is associated with the H-2^b haplotype, and the effect is dominant (Table 2). The H-$2A^b$ allele acts like a typical immune response gene influencing antigen presentation to CD4+ T cells (39). Mice with homozygous mutations in H-2A, such as H-$2A^{bm12}$, or mice with low recovery alleles, such as H-$2A^k$, fail to mount T cell proliferative responses and have a low incidence of recovery (45).

The situation with H-2E is more complex than H-2A because it has both positive and negative effects on FV immunity (35). Mice with an H-2^b haplotype do not express H-2E heterodimers because of a defect in the gene encoding the α chain (46, 47). However, the H-$2E^b$ β gene comes into play when a functional α chain gene is introduced by breeding with mice carrying another haplotype such as H-2^a. H-$2^{a/b}$ heterozygous mice use a hybrid molecule comprised of an E^k α chain and an E^b β chain to present a Friend murine leukemia virus (F-MuLV) envelope peptide to CD4+ T cells (48). Blocking this presentation with specific antibodies reduces recovery, indicating an important positive role in FV immunity (35). However, despite this role, studies in transgenic and MHC recombinant mice have shown that the overall effect of expressing H-2E molecules is a decrease in recovery from FV infection. This reduction in recovery appears to occur through negative thymic selection of T cells that recognize H-2E. Thus, the positive and negative effects are temporally separated with positive effects occurring during the immune response and negative effects occurring during development of the T cell repertoire (Table 2). The H-2Qa-T1a region has a weak, but detectable, effect on recovery from FV leukemia (33). This is a rather large genetic region and the influence on recovery is not very strong, so the exact gene involved has not yet been determined.

In addition to the four MHC genes described above, the immune response to FV is also strongly influenced by a non-MHC gene, Rfv-3 (36). Mice require at least one resistance allele at this locus to make antiviral neutralizing antibodies to clear plasma viremia after FV infection. This effect is necessary, but not sufficient, for recovery from leukemia, as will be discussed further below.

Table 2. MHC class II effects on FV immunity

MHC Class II	Effects on Friend Virus (FV) Immunity
$A^{b/b}$ or $A^{b/k}$	• FV-specific CD4+ T-cell responsiveness • Isotype switching of FV-specific antibodies • Responsiveness to vaccinia/FV envelope vaccination
$A^{k/k}$	• Lack of FV-specific CD4+ T-cell responsiveness • No isotype switching of FV-specific antibodies • Lack of responsiveness to vaccinia/FV envelope vaccination
$E^{b/b}$ (1)	• No thymic selection • No FV-specific CD4+ T-cell responses
$E^{k/b}$ (2)	• FV-specific CD4+ T-cell responses • Negative thymic selection of FV-specific T-cells from the repertoire

(1) H-2E molecules are not expressed on the cell surface in H-2^b mice because of lack of a functional E α gene (46, 47). (2) H-2E α chains form the k haplotype associate with β chains from the b haplotype to form functional cell surface heterodimers that present F-MuLV envelope peptides to CD4+ T cells (48). H-$2E^{k/k}$ molecules may affect the FV-specific immune response also, but their role is unknown.

Studies of FV-Specific Immunity. Several studies have shown significant correlations between recovery from FV leukemia and various parameters of the FV-specific immune response. These include: (*i*) CTL responses, (*ii*) T cell proliferative responses, and (*iii*) production of virus-neutralizing antibodies. Subsequent investigations have established that each of these responses not only correlates with recovery but is also required.

CTL. FV-specific CTL have been shown to recognize antigens in the context of both H-$2D^b$ and H-$2D^d$ molecules (39). The primary CTL response from FV-infected recovering mice is directed against determinant(s) in the F-MuLV envelope protein (49, 50). A peptide from this protein has been described as an epitope for *in vitro* restimulated CTL (51). However, most primary CTL from infected mice do not recognize this epitope, and the major epitope recognized by CTL in recovering mice has not yet been identified. CTL responses also are developed during the rejection of a transplantable Friend tumor cell line, but in contrast to infection with live virus, the predominant CTL response is against an epitope encoded by the viral gag gene (52–56).

$CD8^+$ CTL responses correlate with reduction of splenomegaly in FV-infected animals (49) and probably act by direct killing of infected cells. In the FV model, CTL are detectable by direct assays without *in vitro* stimulation. The *in vivo* importance of the $CD8^+$ T cell response has been demonstrated in resistant H-$2^{b/b}$ mice that were depleted of $CD8^+$ cells before infection with FV. CD8 depletion increases mortality by greater than 70% (49).

T cell proliferation. The rapid development of CD4+ T cell proliferative responses correlates with recovery from a high

dose inoculation of FV (39, 41, 45) (Table 1). The $CD4^+$ T cell response is specific for determinants in the F-MuLV envelope protein (45), and two T helper epitopes from the gp70 portion of envelope have been described at the peptide level (48, 57). One peptide binds to H-$2A^b$ molecules and the other to H-2E molecules, thus providing ligands for recognition by $CD4^+$ T cells. FV-specific $CD4^+$ T cells play a central role in FV immunity, providing immunological help for CTL (50, 58) and B cells (35) and maybe also providing direct antiviral activity. Abrogation of these functions by *in vivo* depletion of $CD4^+$ cells significantly compromises recovery from FV infection (49).

Cytokines. For some murine leukemia viruses, type 1 T helper responses associated with specific cytokine profiles appear protective whereas type 2 responses do not (59). This issue has not been thoroughly addressed in the FV system, but studies on specific cytokines have been done. One study demonstrates depressed IL-2 and tumor necrosis factor-α levels in FV-infected BALB/c mice (60). Furthermore, *in vivo* therapy with tumor necrosis factor-α has been shown to produce temporary regression of FV-induced splenomegaly. However, the mechanism may have been through inhibition of hematopoiesis rather than immunomodulation of FV-specific responses (61). IL-6 and IFNγ levels are depressed in FV-infected DBA/2 mice, and therapy with a combination of IFNγ and lactoferrin increases natural killer (NK) cell activity and enhances survival (62). In other experiments, treatment of FV-infected mice with recombinant human IL-7 was shown to increase NK activity and produced long term survival in 20% of the mice (63). Thus, a major role for cytokines in recovery from FV is likely, but the specific mediators have not yet been completely determined. However, requirements for both CTL and IgG class antibodies in recovery from FV infection suggest that cytokines associated with T_H-1 or T_H-0 type responses might correlate with recovery.

Antibody and B cells. Virus-neutralizing antibodies are required for recovery from FV infection, and their production is influenced by a non-MHC gene, Rfv-3 (Table 3). Rfv-$3^{s/s}$ mice have a suppressed FV-specific antibody response, even in the presence of the proper MHC type (H-$2^{b/b}$) for virus-specific T cell responsiveness. Of interest, Rfv-3 appears to affect only the FV-specific antibody response and not responsiveness to other antigens (64). Failure to mount a virus-neutralizing antibody response to FV infection increases mortality by 90% or greater (58). The Rfv-3 gene has been mapped to chromosome 15 of the mouse, unlinked to the MHC, Ig, or T cell receptor loci (65). However, genetic linkage to several cytokine receptor genes (IL-2Rb, IL-3Rb1, and IL-3Rb2) suggests possible candidates for Rfv-3. It is of obvious interest to elucidate the mechanism by which a retrovirus can specifically suppress the antibody responses directed against it. In addition to the production of virus-neutralizing antibodies, B cells also appear to have important roles in antigen presentation and/or cytokine production. Both $CD4^+$ and $CD8^+$ T cell responses to FV-induced tumors are significantly reduced in B cell-depleted mice (66).

FV-Induced Immunosuppression. FV suppresses both cellular and humoral immune responses in certain strains of mice (64, 67–70), and an important host gene has been mapped to H-2D (42). For example, H-$2D^{d/d}$ mice are susceptible to FV-induced immunosuppression, but H-$2D^{b/b}$ mice are resistant (Table 1). After FV infection in H-$2D^{d/d}$ mice, humoral immune responses to subsequent challenges with strong antigens such as sheep red blood cells are suppressed (70). Responses to T-independent antigens such as 2,4,6,-trinitrophenyl–Ficoll are affected as well, suggesting that immunosuppression need not act through decreased T cell help (64). The involvement of the H-2D region also suggests possible involvement of NK cells. Binding of the Ly-49A receptor on NK cells to H-$2D^d$ molecules can induce global down-regulation of NK cell-mediated killing (71), and decreased NK activity has been associated with FV infection (63). FV-immunosuppressed mice also have been reported to have impaired antigen presentation by macrophages (72). Important to note, susceptibility to immunosuppression does not preclude successful treatment by immunotherapy (58) or protection by vaccination (42).

Immunotherapy. Strain A mice lack virus-specific antibody responses because of their Rfv-$3^{s/s}$ type and fail to recover from FV infection. Immunotherapy using virus-neutralizing mAbs is effective at reducing mortality by 80–100% in A.BY mice, even when treatments are initiated as late as 10 days postinfection (58). Successful therapy requires both $CD4^+$ and $CD8^+$ T cells because depletion of either subset abrogates recovery. In contrast to the success of therapy in A.BY mice, immunotherapy is ineffective in the MHC congenic A strain A/Wy (H-$2^{a/a}$, Rfv-$3^{s/s}$), which is highly susceptible to FV-induced immunosuppression. The cause of the failure of antibody therapy in A/Wy mice appears to be weak T cell responses, which develop with slow kinetics relative to the A.BY strain. However, therapy becomes highly successful in A/Wy mice when the virus inoculum is reduced 5-fold. The resultant slowing of virus spread during antibody therapy allows immune responses to develop before becoming overwhelmed with the viral load. Furthermore, the treated animals are subsequently protected from a high dose challenge of virus. Thus, antibody therapy allows development of long term protective immunity.

Vaccination. Experiments have shown that protection from FV infection can be elicited by several different types of vaccines including killed and attenuated viruses, viral proteins, peptides, and recombinant vaccinia vectors expressing FV genes (73–77). The study of vaccinated mice has allowed the identification of protective immunological epitopes and determination of the types of immunological responses necessary and/or sufficient for protection.

Protective epitopes have been localized to F-MuLV gag and env proteins by using recombinant vaccinia viruses expressing these genes (74, 76). F-MuLV envelope protects against infection better than gag, so most work has concentrated on envelope. The gp70 envelope protein contains at least one CTL epitope (49), three T helper epitopes (48, 57), and two neutralizing antibody epitopes (78–80). The potency of the T

Table 3. Recovery from FV induced leukemia is influenced by MHC genes (H-2) and Rfv-3

Mouse strain	H-2	Rfv-3†	Day 30 postinfection* FV viremia	Day 30 postinfection* FV neutralizing antibody	Recovery from FV leukemia
A.BY	b/b	s/s	+	−	no
(C57BL/10 × A.BY)F1	b/b	s/r	−	+	yes
A/WySn	a/a	s/s	+	−	no
(B10.A × A/WySn)F1	a/a	s/r	−	+	no

*All of these mouse strains have similar levels of viremia at 10-14 days postinfection with FV.
†s/r mice are similar to r/r mice in recovery from viremia and antibody production.

helper determinants has been demonstrated by successful vaccination with a small envelope peptide containing a T helper cell epitope (77).

Protection from FV-induced disease in vaccinated mice correlates with antibody responses, $CD4^+$ T cell proliferative responses, and $CD8^+$ CTL responses (74, 76, 81). Of interest, the requirement for $CD8^+$ T cells in protection is dependent on the number of T helper epitopes in the vaccine (50). Mice immunized with a recombinant vaccinia vector expressing the full length F-MuLV envelope protein containing multiple immunological epitopes require $CD4^+$ T cells for protection but not $CD8^+$ T cells. However, if the number of immunological epitopes in the vaccine is reduced, $CD8^+$ T cells as well as $CD4^+$ T cells are critical for protection. Surprisingly, $CD8^+$ T cell epitopes are not necessary in the vaccine even when $CD8^+$ T cells are required for protection. This paradox appears to be due to the ability of vaccine-primed $CD4^+$ T cells to provide immunological help for $CD8^+$ T cells that are stimulated by the live virus challenge. Additional data also indicate that the expression of multiple CD4 epitopes in the vaccine is more important than expression of CD8 epitopes (50).

The method of immunization can dramatically alter the efficacy of vaccination, especially in terms of the ability to cross-protect different strains of mice. For example, immunization by tail scratch with recombinant vaccinia expressing the F-MuLV env protein protects $H\text{-}2^{a/b}$ mice but not MHC congenic $H\text{-}2^{a/a}$ mice (74) (Table 4). The nonresponsiveness of $H\text{-}2^{a/a}$ mice maps to the H-2A class II genes (42). On the other hand, when the same protein is biochemically purified and inoculated s.c. with complete Freund's adjuvant or synthetic adjuvants, both strains of mice are protected (81, 82) (Table 4). Thus, there does not appear to be a complete lack of envelope responsive immune cells in $H\text{-}2^{a/a}$ mice, but their responsiveness is weak in the absence of adjuvant. Immunization with a live attenuated virus also protects mice of several MHC types, including $H\text{-}2^{a/a}$ mice (42, 74). The ability to protect regardless of MHC type correlates with induction of detectable, cell-mediated, and neutralizing antibody responses before challenge (74). Thus, the virus is faced with preexisting immunological effectors that can reduce the effective virus dose.

Implications. In conclusion, the FV model has yielded valuable information regarding genetic resistance to retroviral disease, but it is obvious that much remains to be discovered about the immunological mechanisms by which the genes impart their influence. Of particular interest are how the Rfv-3 gene causes susceptibility to suppression of the FV-specific antibody responses, how class I MHC genes influence FV-specific $CD4^+$ T cell proliferative responses, and how the H-2D gene influences virus-induced general immunosuppression. The elucidation of these mechanisms may aid in the development of immunotherapies and vaccines that may be applicable to human diseases.

Although results from FV studies cannot be directly related to human infections such as HIV, consideration of human data in light of the FV results may lead to new interpretations and even better designs for human experiments. For example, it is now known that both non-MHC (83–85) and MHC genes (86) influence the rate of HIV infection and progression to AIDS in humans. Furthermore, there is no reason to suspect that the immune responses required to deal with HIV would be any less complex than those illustrated for FV in mice. Thus, by analogy with the results of FV immunotherapy, part of the reason for the failures of passive antibody therapies in AIDS patients may be related to the high virus loads and low T cell counts in the patients studied (87–95). The FV results suggest that HIV immunotherapy might be more successful if initiated early during the course of infection before virus-induced $CD4^+$ T cell depletion.

The best hope for controlling the worldwide pandemic of AIDS lies in development of an effective vaccine. One message that might be gleaned from the FV experiments is that a successful HIV vaccine would most likely be one that stimulates multiple immune system components with a broad spectrum of antigens. Priming with multiple $CD4^+$ T cell epitopes might be very important because of the central role these cells play in amplifying both CTL and antibody responses. One of the best FV vaccines is the live attenuated virus, and live attenuated viruses have been the most successful vaccines in the simian immunodeficiency virus model as well (18). However, there are several concerns about using such a vaccine for HIV in humans. These include reversion to virulence, insertional mutagenesis, recombination with endogenous retroviral sequences to produce new infectious viruses, and pathogenesis in immunocompromised hosts. Ideally, one might construct a live nonretroviral vector to deliver HIV antigens that would replicate for longer periods of time than recombinant vaccinia and still avoid the major drawbacks of retroviral vectors cited above. Continuous expression over a 2- to 3-week period would more closely mimic immunization by a live attenuated retrovirus and allow development of potent immune effectors. Optimal retroviral protection may require the presence of specific effectors rather than just immunological memory, so further studies will be required to determine how such effectors can be persistently maintained.

Table 4. Protection of mice with different MHC types using various vaccines

MHC genotype	Protection from FV challenge after vaccination		
	Vaccinia/FV env*	FV envelope in adjuvants†	FV-N‡
$H\text{-}2^{b/a}$	+	+	+
$H\text{-}2^{a/a}$	−	+	+

*Vaccinia recombinant expressing F-MuLV envelope (74).
†Complete Freund's adjuvant or synthetic adjuvants (81, 82).
‡FV-N replicates poorly in $Fv\text{-}1^{b/b}$ mice and acts as an attenuated live virus (74).

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Non-Human Primate Models for AIDS Vaccine Research

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Abstract: Since the discovery of simian immunodeficiency viruses (SIV) causing AIDS-like diseases in Asian macaques, non-human primates (NHP) have played an important role in AIDS vaccine research. A multitude of vaccines and immunization approaches have been evaluated, including live attenuated viruses, DNA vaccines, viral and bacterial vectors, subunit proteins, and combinations thereof. Depending on the particular vaccine and model used, varying degrees of protection have been achieved, including prevention of infection, reduction of viral load, and amelioration of disease. In a few instances, potential safety concerns and vaccine-enhanced pathogenicity have also been noted. In the past decade, sophisticated methodologies have been developed to define the mechanisms of protective immunity. However, a clear road map for HIV vaccine development has yet to emerge. This is in part because of the intrinsic nature of the surrogate model and in part because of the improbability of any single model to fully capture the complex interactions of natural HIV infection in humans. The lack of standardization, the limited models available, and the incomplete understanding of the immunobiology of NHP contribute to the difficulty to extrapolate findings from such models to HIV vaccine development. Until efficacy data become available from studies of parallel vaccine concepts in humans and macaques, the predictive value of any NHP model remains unknown. Towards this end, greater appreciation of the utility and limitations of the NHP model and further developments to better mimic HIV infection in humans will likely help inform future AIDS vaccine efforts.

Key Words: Non-human primates, live attenuated virus, prime-boost, SHIV, HIV, SIV.

INTRODUCTION

Successful vaccines made to date are primarily against pathogens that can induce protective immunity as a result of natural exposure. Well known examples include smallpox, polio, and measles. Survivors of natural infections develop life-long immunity against disease upon re-exposure. In fact, the observation that immunity can be acquired as a result of natural exposure formed the basis for the practice of active immunization, beginning with variolation in centuries past and continuing with vaccinations in modern history [1]. In the case of HIV infection, evidence for protective immunity acquired from natural infection is far from clear. Cytotoxic T-lymphocyte (CTL) responses have been implicated in the control of virus replication in the acute phase of HIV infection [2,3]. Preservation of T-helper cell functions correlates with better clinical outcome [4,5]. Substantial HIV-specific antibody and CTL responses can be generated by infected individuals, but they are ineffective in controlling infection, as escape variants eventually take over [6-8]. Significantly, CTL and proliferative and mucosal IgA responses have been detected in rare cases of uninfected partners of infected individuals and multiply-exposed seronegative individuals [9-12]. Whether these responses account for the control of infection remains unclear. On the other hand, there is mounting evidence for superinfection in HIV-positive individuals [13-16], indicating the absence of protective immunity from natural exposure. In short, data available to date do not support the notion of naturally acquired immunity against HIV infection and diseases, as has been observed in many vaccine-preventable diseases.

So far, the only direct evidence supporting the feasibility of inducing protective immunity against primate lentiviruses has come from non-human primate (NHP) models. A number of vaccine strategies and immunization approaches have shown protection against infection or diseases. Recent studies have shed important insights on the potential correlates of protection, but also on the significant obstacles yet to be overcome. However, because of the complexity and limitations of the NHP models, it remains difficult to extrapolate data from these models to inform the development of HIV vaccines. As a result, the utility of NHP models in HIV vaccine development has been debated. This article reviews some of the underlying issues and proposes potential directions that may result in more effective use of NHP models for HIV vaccine research. The reader is referred to a number of excellent articles that provide a more in-depth review of the NHP models, summation of vaccine trials in NHP, and discussions on the pros and cons of specific vaccine approaches [17-24; http://hiv-web.lanl.gov/cgi-bin/vaccine/public/index.cgi].

NON-HUMAN PRIMATE MODELS FOR AIDS

HIV-1 and HIV-2

The search for an animal model for AIDS started soon after the discovery of HIV-1 as the etiologic agent. Alter *et al.* [25] reported seroconversion and transient lymphadenopathy in chimpanzees inoculated with plasma from HIV-infected patients. However, with the exception of a few iso-

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lated cases resulting from serial passages [26,27], HIV infection in chimpanzees generally does not lead to AIDS. Evidence indicating chimpanzees being the natural host of an endemic virus SIVcpz, a likely predecessor of HIV-1 [28], may explain the lack of pathogenic responses. In any case, the endangered species status of chimpanzees coupled with restricted availability and high costs prohibit the general use of this animal model for AIDS research.

HIV-1 infection in pig-tailed macaques was attempted in the early 1990's [29]. However, infection was transient and sporadic. Even though serial passages in neonates resulted in enhanced replication and durable antibody responses, no evidence of $CD4^+$ T-cell depletion was observed [30]. Recently, several host restriction factors for HIV-1 replication have been identified. Macaque TRIM-5α, a component of cytoplasmic bodies, blocks HIV-1 replication at a step after viral entry, prior to reverse transcription [31]. The action of APOBEC3G, a single strand DNA-editing enzyme inducing hypermutation and DNA degradation, can be counteracted by the viral *vif* gene product [32]. Further understanding of species-specific restriction factors and their interactions with viral protein targets may point to new approaches to adapt HIV-1 for more efficient replication in macaques.

HIV-2 is believed to have evolved as a result of cross-species transmission of SIVsmm, a lentivirus endemic to some sooty mangabey populations in Western Africa [33]. Because of its close relatedness to SIVsmm, HIV-2 infection of NHP was explored as a model for AIDS. Early efforts resulted in mostly transient infections [34,35]. Upon repeated passages, several HIV-2 strains have been adapted in baboons [36,37] and pig-tailed macaques [38,39] that are capable of inducing persistent viremia, rapid $CD4^+$ T-cell decline, and AIDS. However, HIV-2 models have not been widely used for HIV vaccine research, perhaps in part because of their similarity to SIV and in part because of the focus on HIV-1. Nevertheless, it should be noted that HIV-2 infection of baboons or pig-tailed macaques provides the only models for AIDS pathogenesis based on a virus of human origin, rather than SIV or SIV/HIV chimera, SHIV (see below). HIV-2 isolates, including HIV-2/287, can utilize CXCR4 as the co-receptor [40-42], a feature shared with HIV-1, but not with SIV. The basis for this difference is not known, but may be related to adaptation in humans. In this sense, HIV-2 models may provide unique advantages for vaccine and pathogenesis studies not previously appreciated.

SIV and SIV/HIV Chimera

SIV was isolated in the early 1980's from monkeys with AIDS-like diseases or lymphoma [21,43-45]. According to the species from which it was first isolated, it has been designated SIVmac (from rhesus macaques), SIVsmm (from sooty mangabeys), or SIVmne (from pig-tailed macaques, *Macaca nemestrina*). These isolates share a common ancestor, SIVsmm, a virus that is endemic and generally non-pathogenic in its natural hosts, sooty mangabeys [33]. Experimental inoculation of SIV into a number of Asian macaque species, including rhesus, pig-tailed and cynomolgus monkeys, results in a spectrum of pathological responses similar to AIDS in humans. Because of its ability to cause AIDS-like diseases in relatively accessible primate species, SIV infection of macaques has been the animal model of choice for AIDS vaccine research.

Several key findings establish the similarities between SIV infection of macaques and HIV-1 infection of humans. Like most HIV-1 isolated from early infection [46,47], the majority of SIV isolates examined to date utilize the CCR5 coreceptor for viral entry [41,48-50]. Infection by SIV is characterized by massive, rapid, and selective depletion of memory T cells in gut-associated lymphoid tissues, a finding later confirmed in HIV infection [51-56]. Both viruses replicate not only in activated and proliferating T cells, but also resting T cells [57]. Acute infection in HIV-1 and SIV models resolves with the onset of antigen-specific immune responses [2,3,58-61]. Both viruses utilize similar evasion tactics to escape from host immune responses, including modification of glycosylation patterns in viral envelope protein [62-65] and mutations in neutralization and CTL determinants [6,7,66]. Importantly, in both HIV and SIV infections, plasma viral load after the acute phase ("viral setpoint") predicts the rapidity of disease progression [67-69]. Peripheral blood $CD4^+$ T-cell depletion often precedes the onset of AIDS-defining events (e.g., opportunistic infections, neoplastic diseases; hematological and neurological disorders), although the duration of disease-free periods differs significantly between HIV-1-infected humans and SIV-infected macaques (an average of 8-10 years for humans *vs.* 0.5-3 years for macaques infected with the majority of pathogenic SIV strains). These features common between HIV and SIV infections define the unique advantage of the SIV model for the study of HIV pathogenesis.

On the other hand, the SIV model also has a number of shortcomings. First, by its very nature, SIV infection of macaques only provides a "surrogate" model for HIV infection. SIV shares approximately 80% genomic sequence homology with HIV-2, but only 40-50% with HIV-1 [70]. Serological cross-reactivity between SIV and HIV-1 is limited [71]. Efficacy of HIV-1-based vaccines, therefore, cannot be directly evaluated in the SIV model. Second, most of the commonly used SIV isolates have been multiply passaged in macaques to select for increased virulence and rapid disease progression [23,72]. The basis for the increased virulence is not clear, but is likely related to the accumulation of mutations in multiple regions of the viral genome (e.g., *gag* and *env*) and the acquisition of CTL-escape and neutralization-resistance phenotypes [7,66,73]. Viruses with enhanced virulence may allow for a more rapid and uniform determination of challenge outcome in vaccine studies with few animals. However, the relevance of these viruses to HIV infection is not clear, and the reliance on these models for challenge studies may underestimate vaccine efficacy. Third, the choice of macaque species or genotype also needs to be considered. Using animals with defined genotypes, such as rhesus macaques with Mamu-*01 and Mamu-B*17 major histocompatibility complex (MHC) I alleles, may provide a more uniform outcome than non-MHC-matched animals, but may also bias the result because the allele has been linked to better disease outcome after SIVmac239 infection [74,75]. Infection in rhesus macaques of Chinese origin is characterized by lower viral load and less pronounced $CD4^+$ T cell deple-

tion than those of Indian origin [76-79]. Similarly, infection in cynomolgus macaques (*Macaca fascicularis*) appears to be less virulent than infection in Indian rhesus, with plasma viral loads more compatible with typical HIV infection in humans [80]. Finally, differences between experimental inoculation of animals and natural transmission in humans also need to be considered. Most, if not all, current models rely on the use of cell-free virus as inoculum. It is not clear to what extent this provides an adequate model for natural transmission, which likely involves both cell-free and "cell-associated" viruses. The commonly used intravenous route of inoculation is highly reproducible and is a reasonable mimic of blood-borne HIV transmission. However, other than experimental inoculation at mucosal sites (intrarectal, intravaginal, oral), there is currently no established macaque model for sexual transmission. Since vaccine studies are usually limited by the availability of animals, mucosal inoculations generally employ relatively high doses of cell-free virus inoculum to achieve uniform infection. The relevance of such models has been debated, since natural sexual transmission through intact mucosa appears to be a low probability event [81]. In this context, a low-dose, repeated mucosal exposure model may offer a useful alternative [82].

To address the need for direct testing of HIV vaccines in an animal model, chimeric viruses were developed, in which the *tat, rev, vpu* and *env* genes of HIV-1 were inserted into the genome of the pathogenic molecular clone of SIVmac239 [83-87]. Inoculation of macaques with these chimera resulted in persistent infection [85,87-89] and, upon serial *in vivo* passages, rapid $CD4^+$ T-cell depletion, followed by AIDS-like diseases [90,91]. SHIV shares many of the advantages of SIV macaque models. In addition, it allows direct testing of Env-based HIV-1 vaccines. However, there are also significant differences between commonly used SIV and the SHIV strains. For example, infection of SHIV89.6P results in rapid depletion of peripheral blood $CD4^+$ T-cells (generally within 2-4 weeks) [91], in contrast to the gradual decline observed in most SIV and HIV-1 infections. SHIV89.6P utilizes CXCR4 for infection, unlike SIV and most HIV-1 early isolates, which utilize CCR5 [41]. The difference in co-receptor usage is reflected in target cell populations after infection and the disease course that follows. Harouse *et al.* [92] observed that a CCR5-tropic virus, SHIV162P, caused a profound loss of $CD4^+$ T-cells in the intestine, not in the periphery, whereas the opposite was observed for a CXCR4-tropic virus, SHIV33A. Nishimura *et al.* [93] reported that a CXCR4-using SHIV, DH12R, targets naïve T-cells, resulting in rapid $CD4^+$ T-cell loss in the periphery, whereas SIVmac239 primarily targets CCR5-expressing memory $CD4^+$ T-cells. SHIV89.6P is also relatively sensitive to neutralizing antibodies [91,94], whereas SIVmac239 is highly resistant [95]. Proper interpretation of vaccine efficacy data will require in-depth understanding of the biological properties of the challenge models used [96]. Currently, there is only one established SHIV challenge model, SHIV162P, that is based on a CCR5-using virus. However, the significant variations in setpoint viral load, and the gradual and variable decline of $CD4^+$ T-cells in the periphery [97] make it difficult to rely on these parameters as indicators of vaccine protection. Obviously, further development and refinement of SHIV models are needed.

PROTECTIVE IMMUNITY AGAINST HIV/AIDS: INSIGHT FROM NHP STUDIES

NHP models have been used to evaluate the safety, immunogenicity and protective efficacy of multiple vaccine approaches. Perhaps one of the most important insights gained from these studies is the feasibility of immune protection against primate lentivirus infection and disease. As it is beyond the scope of this article to review all the vaccine approaches tested in NHP models, the discussion below will focus on those that have shown general applicability and protective immunity in multiple models.

Live attenuated vaccine, as exemplified by *nef*-deleted mutant SIVmac239Δnef, has been shown to protect against challenge by highly pathogenic cloned virus SIVmac239, or uncloned SIVmac251 in rhesus macaques [98]. Maximal protection was reached 6-10 months after vaccination, possibly due to the need for immune responses to mature [99-101]. On the other hand, protection has also been observed in macaques as early as 21 days after vaccination [102]. In this case, protection did not correlate with any specific T-cell or antibody responses measured [103]. The potential role of viral interference or competition for target sites needs to be examined. Efficacy of live attenuated vaccine appears to depend on the replicative capacity of the vaccine virus, as multiply deleted virus SIVmac239Δ3 [101,104], or tissue culture-passaged virus SIVmac1A11 [105], afforded only partial or little protection. It is also important to note that protection induced by live attenuated virus vaccine was primarily effective against the homologous virus and was significantly reduced against a heterologous pathogenic virus, SIVsmE660 [106]. Furthermore, the live attenuated virus approach has been associated with significant safety concerns that are likely to preclude the development of similar HIV vaccines for the general population in the foreseeable future. SIVmac239Δnef showed no attenuation in newborn macaques [107]. Disease progression in adult macaques was delayed, but not abrogated [108-110]. There are also theoretical risks associated with the ability of retroviruses to integrate into the host chromosome [111]. Finally, without an appropriate animal model for HIV-1 pathogenicity, it is difficult to assess the safety of candidate live vaccines. Nevertheless, live attenuated vaccines may serve as an excellent model to study HIV pathogenesis and correlates of protection against primate lentiviruses.

The use of different vaccination approaches for priming and boosting ("prime-boost") was originally explored as a means to overcome anti-vector immunity elicited against the priming immunogen and to augment antigen-specific responses by subunit protein boost [112,113]. This approach was found to enhance antigen-specific responses in mice, macaques, and humans primed with a recombinant vaccinia virus and boosted with recombinant HIV-1 envelope protein [112, 114-117]. Protective efficacy of this "prime-boost" approach was first demonstrated in a moderately pathogenic SIVmne model against both intravenous and mucosal infection [114,117-119]. Inclusion of multiple antigenic targets (e.g., envelope and core antigens) in the vaccine design augmented the breadth of protection against uncloned virus challenge [120]. A poxvirus and protein prime-boost regi-

men also protected against SHIV IIIB challenge in pig-tailed macaques [121]. On the other hand, Giavedoni *et al.* [122] and Daniel *et al.* [123] reported that immunization with a similar prime-boost regimen resulted only in reduction of viral load in a minority of animals challenged with a highly pathogenic virus, SIVmac251, with no apparent benefit in disease outcome. Abimiku *et al.* [124] showed that macaques immunized with recombinant canarypox vaccines and boosted with subunit HIV-1 proteins were partially protected against infection by a divergent but non-pathogenic HIV-2. Hirsch *et al.* [125] showed that immunization with a modified vaccinia Ankara (MVA) expressing multiple SIV antigens followed with inactivated SIV failed to protect against infection by a more pathogenic challenge virus, SIVsmE660, but was able to reduce virus load resulting in prolonged disease-free survival. It therefore appears that immune responses elicited by these early attempts at virus vector priming and protein boosting were suboptimal, sufficient to protect against challenge virus of low pathogenicity, but failed to contain more robust ones. It is noteworthy that Patterson *et al.* [126] achieved protection against mucosal challenge by a highly pathogenic virus, SIVmac251, using replication-competent adenovirus for priming and subunit proteins for boosting. This result lends further support for the continued investigation of the vector-protein "prime-boost" strategy for immunization.

Other "prime-boost" strategies have also been explored. In particular, DNA priming with recombinant virus boosting was found to elicit strong T-cell responses [127,128]. Significant and sustained reduction of viral load has been achieved by DNA/MVA prime-boost against CXCR4-using SHIV [129,130]. But its efficacy against SIVmac251 or SIVmac239 challenge was much less impressive [131-133]. Similarly, replication-defective adenovirus vector, alone or as a booster to DNA priming, elicited robust T-cell responses and significant reduction of viral load after SHIV89.6P challenge [134,135]. DNA prime with recombinant Sendai virus boost has protected cynomolgus macaques against SHIV89.6PD challenge (136,137). Protection by DNA prime and recombinant attenuated *Listeria monocytogenes* boost was recently reported [138]. The order of DNA versus recombinant vector for priming or boosting was examined. Contrary to earlier observations of McMichael and colleagues [127,128], priming by recombinant poxvirus followed by DNA boost is at least as effective as the reverse order for eliciting protective immunity [139]. Whether this difference relates to the properties of replication-competent *vs.* non-replicative poxvirus vectors remains unclear. Although immunity elicited by DNA alone is relatively weak, it potentiates responses to booster immunization by recombinant vectors [130,131,134,139,140]. In this sense, current methods of measuring immune responses may not be sufficient to fully reveal the action of priming. In part because of the disappointing results obtained to date with DNA vaccines in humans and in part because of the need to circumvent anti-vector immunity elicited by priming vectors, increasing efforts have been focused on heterologous vectors for prime-boost. Ramsberg *et al.* [141] reported that prime-boost with attenuated recombinant vesicular somatitis virus (VSV) and recombinant MVA elicited substantially better responses and protective immunity against SHIV89.6P challenge than repeated immunizations with recombinant VSV of different serotypes. Triple combination prime-boost with DNA, recombinant Semliki Forest virus and MVA vectors has also shown protective immunity in cynomolgus macaques against SIVmac251 [142]. Other heterologous vector prime-boost strategies are sure to follow [e.g., 143].

DNA priming followed by protein boosting has been found to be effective to induce antibody responses [144-147]. With the reemerging emphasis on vaccines that can elicit neutralizing antibodies, DNA-protein prime-boost is increasingly being used as a platform to evaluate novel antigen designs. Because protection against primate lentiviruses most likely will require both the humoral and cellular arms of host immune responses, systematic evaluation of various prime-boost approaches appears to be necessary. So far, ample evidence has been accumulated supporting the notion that heterologous prime-boost approaches can elicit greater immune responses than single immunization modalities. However, the mechanism underlying such enhanced response is not well understood [131,148,149]. Detailed analysis of the role of innate immunity and the development of adaptive responses by systematic and comparative prime-boost studies may help identify optimal approaches to enhance protective immunity. Finally, even though prime-boost approaches have shown promise, they also have significant shortcomings, including the need to manufacture multiple vaccine components (usually on diverse technical platforms) and to comply with complex immunization schedules. As in all combination approaches, the potential for increased side effects also needs to be considered.

Studies in NHP models have also helped define the correlates of protection against primate lentiviruses. The most definitive information has been obtained from passive transfer of neutralizing antibodies. Early studies by Emini *et al.* [150] showed that neutralizing antibody directed to the V3 loop of HIV-1 protects chimpanzees against infection by a T-cell line-adapted (TCLA) virus, HIV-1 IIIB. However, the implication of this finding for vaccine development has been debated because of the discovery that primary isolates of HIV-1 differ significantly from TCLA viruses in their neutralization sensitivity [151,152] and the observation that the V3 loop sequence is highly variable. Nevertheless, results from a number of studies have firmly established that passively transferred neutralizing antibodies, monoclonal or polyclonal, when present in sufficient quantity, can protect macaques against both CXCR4- and CCR5-using SHIV [153-158]. So far, none of the vaccine approaches tested can elicit neutralizing antibody responses comparable with those needed to achieve protection in passive transfer studies [157, 159]. Therefore it remains a major goal in AIDS vaccine research to design immunogens that elicit robust and broadly neutralizing antibody responses. It is intriguing to note that passively transferred neutralizing antibodies given within 24h post-infection can delay disease significantly [160]. It appears that the presence of neutralizing antibodies during acute viremia can accelerate the development of an effective humoral response. Several challenge studies have shown that neutralizing antibody detection is accelerated in vaccinated macaques [139,161]. Therefore, it is also important to examine if sub-optimal neutralizing antibodies, together with

recall responses and cell-mediated immunity elicited by active immunization, will suffice to afford protection.

Although the outbred nature of macaques limits the use of passive transfer experiments to demonstrate directly the role of cell-mediated immunity in protection, it is clear that such responses are of critical importance. Selective depletion of T-cell subsets and correlative studies have established the importance of antigen-specific $CD4^+$ and $CD8^+$ T cell responses in control of virus replication [2,3,58-61,162]. However, there is as yet no consensus on any single or combination of parameter(s) to measure T cell responses that are predictive of vaccine protection in NHP. Multiparametric analysis that measures multiple phenotypic markers and functional responses [163] may be necessary. Furthermore, studies in NHP have also revealed the possible importance of balanced immune responses. Induction of antigen-specific $CD4^+$ responses in the absence of functional $CD8^+$ responses has been suggested as the possible reason for the apparent enhancement of infection in immunized macaques after challenge [164,165]. CD8-mediated antiviral factors have been identified in HIV-1-infected individuals and have been shown to be highly effective in blocking infection by primary virus isolates [166]. However, current knowledge is still insufficient to fully define the nature of the anti-viral activity and to determine if and how such responses can be elicited by vaccination. In this regard, studies of innate responses in the context of vaccination and challenge infection should receive greater attention.

LIMITATIONS AND FUTURE DIRECTIONS

Although substantial knowledge has been gained from NHP models, it is not necessarily straightforward to extend these findings to inform HIV vaccine development. The controversy surrounding the failed efficacy trial of gp120 subunit protein vaccines may serve to illustrate this point. Since this vaccine has been shown to elicit neutralizing antibodies and protect chimpanzees against HIV-1 IIIB challenge [167], the failure of this vaccine in human trials [168,169] has been viewed as evidence to invalidate NHP models. While this view may be justified as far as the HIV-1 IIIB challenge model in chimpanzees is concerned, key findings from NHP models as a whole are remarkably consistent with the results from human trials. First, although gp120-elicited antibodies neutralized TCLA viruses and other highly sensitive isolates (e.g., HIV-1 SF2), sera from immunized chimpanzees and humans failed to neutralize typical primary HIV-1 isolates [151,152]. In this sense, chimpanzees are suitable for immunogenicity assessment, but not for challenge studies. Second, a similar SIV envelope protein vaccine failed to elicit neutralization antibodies and to protect macaques against SIVmac251 infection [122]. Thus, available data from both NHP models are consistent with the outcome of human efficacy trials. In other words, for a vaccine that bases its mode of action primarily on neutralizing antibodies, protection can be achieved if sufficient neutralizing antibodies are present (as in HIV-1 IIIB infection of chimpanzees), but not when they are lacking (as in SIV models and in humans). Proper interpretation of findings from NHP models therefore requires better understanding of the characteristics and the limitations of the models used.

As discussed in previous sections, a key limitation of the NHP model is its intrinsic nature as a surrogate model for HIV infection. SIV models do not allow direct testing of HIV vaccines. Currently available SHIV models do not adequately represent the spectrum of HIV genotypes and phenotypes. In particular, very few CCR5-using and non-subtype B SHIV are available as challenge stocks. Selection for increased virulence by serial passage in macaques may be useful for rapid and reliable read-out of challenge outcome, but may also result in misjudgment of vaccine efficacy. Recently, several host restriction factors for HIV-1 replication in macaque cells have been identified [31,32]. If the nature of host restriction and the target sites on the virus can be identified, it may be possible to introduce limited and specific alterations in HIV-1, enabling it to replicate more efficiently in macaque cells and establish persistent infection *in vivo*. The availability of such challenge viruses may allow direct testing of HIV-1 vaccines in a more relevant model. Until then, currently available surrogate models are best suited for understanding the basic biology of immune protection and testing of vaccine concepts, not necessarily vaccine products *per se*.

As illustrated by the example of gp120 trials discussed above, another difficulty to extract information from NHP models is the seemingly contradictory findings from different models. Several factors may contribute to this. At the most basic level, there is a lack of comparability and standardization of reagents, methods and challenge stocks, making it difficult to compare data from different vaccine studies. Better standardization of reagents and comparability of experimental design is urgently needed and is only possible through a concerted effort. On another level, the apparent discordance could be a reflection of the different properties of the challenge model used. For instance, immune responses required for protection against a neutralization-sensitive virus, such as SHIV89.6P, will most likely be different from that for a neutralization-resistant one, such as SIVmac239. Since HIV-1 infection in humans results in a wide spectrum of responses and outcomes, it is doubtful any single model will adequately recapitulate such complexity. Future efforts will most likely rely on models that reflect the range of HIV-1 infection, in terms of viral genotype and phenotype, as well as the mode of transmission. Finally, intrinsic differences between NHP species and humans may also contribute to discordant findings. In addition to their varying susceptibility to virus infection, different species may recognize immunogens and respond to adjuvants differently. For example, species specificity of the adjuvant activities of bacterial lipopolysaccharides and CpG oligonucleotides has been well recognized [170-172]. Proper interpretation of discordant results from different species will require better understanding of the mechanisms of action of the immunogens and adjuvants involved.

The predictive value for any animal model depends on validating data from human trials. The lack of efficacy data from human vaccine trials to date makes it risky to select of any single NHP model to "rank-order" candidate vaccines for clinical development. On the other hand, it is not feasible and is scientifically unsound to screen all experimental vaccines in early phase human trials. Judicious use of appropri-

ate NHP models will greatly accelerate AIDS vaccine development. Towards this end, better understanding of the basic biology of NHP models, development of models that better reflect HIV in natural transmission, and greater emphasis on comparative and parallel-track studies in humans and NHP are critically needed.

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Simian Immunodeficiency Virus Infection of Monkeys as a Model System for the Study of AIDS Pathogenesis, Treatment, and Prevention

I. Introduction

Simian immunodeficiency viruses (SIV) are a large family of primate lentiviruses that naturally infect a wide range of African primates. These viruses are highly relevant models for the study of human AIDS since upon experimental infection of macaques, they induce an immunodeficiency that is remarkably similar to AIDS in humans. This has lead to extensive characterization of a number of isolates of SIV which are presently used in the study of AIDS pathogenesis, the development of vaccines, and the assessment of antiviral therapies. An essential component of these animal studies has been the use of plasma viral RNA assays for assessing viral replication. This chapter reviews the relative pathogenicity of different isolates of SIV and discusses the use of plasma viremia as an early readout for the study of pathogenesis, therapy, and vaccine development.

At the present time, these primate lentiviruses can be classified into five lineages based upon sequence and functional genetic organization. These

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five lineages are represented by (1) SIVcpz from chimpanzees (*Pan troglodytes*), (2) SIVsm from sooty mangabeys (*Cercocebus torquatus atys*), (3) SIVagm from four species of African green monkeys (members of the *Chlorocebus aethiops* superspecies), (4) SIVsyk from Sykes' monkeys (*Cercopithecus mitis albogularis*), and (5) SIVmnd from a mandrill (*Mandrillus sphinx*) together with SIVlhoest from l'hoest monkeys (*Cercopithecus l'hoesti lhoesti*) (Hirsch *et al.*, 1999) and SIVsun from sun-tailed monkeys (*Cercopithecus l'hoesti solatus*) (Beer *et al.*, 1999). More detailed information on the phylogenetic relationships between these viruses have been reviewed previously (Franchini and Retiz, 1994; Hirsch and Johnson 1993; Johnson and Hirsch, 1993; Sharp *et al.*, 1995). Recently SIVrcm from red-capped mangabeys (SIVrcm; *Cercocebus torquatus torquatus*) was partially characterized by Georges Courbot *et al.* (1998) and SIVdrl from drills (SIVdrl; *Mandrillus leucophaeus*) was partially characterized by Clewley *et al.* (1998). Complete analysis of their genomes will be required to determine whether these are representative of new lineages. The various SIV strains are listed in Table I and the phylogenetic relationship between fully characterized

TABLE I Major Lineages of Simian Immunodeficiency Virus

SIV strain	*Species of origin*	*Scientific name*
SIVsm	Sooty mangabey	*Cercocebus atys*
SIVmac	Macaque	*Macaca sp.*
SIVstm	Stumptailed macaque	*Macaca arctoides*
SIVmne	Pigtailed macaque	*Macaca nemestrina*
HIV-2	Human	*Homo sapiens*
SIVagm	African green monkey	*Chlorocebus aethiops sp.*
SIVagm/ver	Vervet monkey	*Chlorocebus aethiops pygerythrus*
SIVagm/gri	Grivet monkey	*Chlorocebus aethiops aethiops*
SIVagm/tan	Tantalus monkey	*Chlorocebus aethiops tantalus*
SIVagm/sab	Sabaeus monkey	*Chlorocebus aethiops sabaeus*
SIVsyk	Sykes' monkey	*Cercopithecus mitis albogularis*
SIVl'hoest	L'hoest monkey	*Cercopithecus l'hoesti*
SIVsun	Sunrailed monkey	*Cercopithecus solatus*
SIVmnd	Mandrill	*Mandrillus sphinx*
SIVrcm	Redcapped mangabey	*Cercocebus torquatus torquatus*
SIVdrl	Drill	*Mandrillus leucophaeus*
SIVcpz	Chimpanzee	*Pan troglodytes*
HIV-1	Human	*Homo sapiens*

lineages of SIV and HIV is depicted in Fig. 1. Each of the lineages share approximately 50% identity between the most highly conserved *gag* and *pol* genes. The identification of unique but related SIV isolates within the members of the African green monkey lineage implies that these viruses are ancient, since speciation is estimated to have occurred many thousands of years ago. It is therefore believed that the SIVs coevolved with their host species. A similar situation has been observed recently in members of the l'hoesti superspecies (l'hoest monkeys and sun-tailed monkeys) (Beer *et al.*, 1999).

In addition to this evidence of long-term evolution within African primates, there are situations that can only be explained by recent cross-species transmission (reviewed in Sharp *et al.*, 1995). For example, a remarkable phylogenetic relationship exists between SIV isolated from sooty mangabey monkeys (SIVsm; *Cercocebus torquatus atys*) and HIV-2 in West African humans (Hirsch *et al.*, 1989; Marx *et al.*, 1991; Gao *et al.*, 1992; reviewed in Sharp *et al.*, 1995). Indeed, some of these viruses cannot be distinguished phylogenetically, implying that HIV-2 arose by cross-species transmission

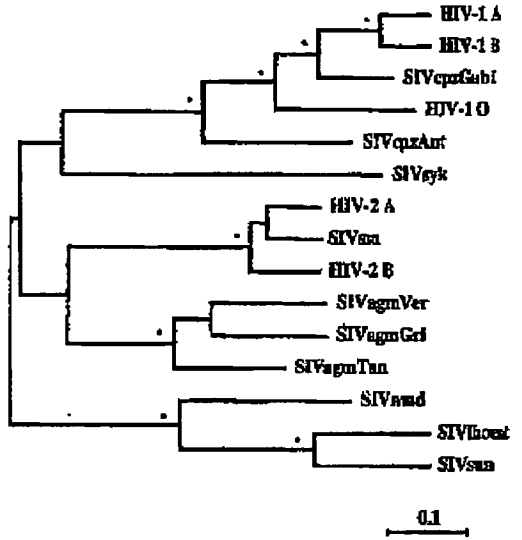

FIGURE 1 The phylogenetic relationship between representative HIV and SIV strains is shown in this maximum-likelihood analysis of concatenated Gag-Pol-Vif-Env-Nef proteins. Five SIV lineages are represented respectively by SIVcpz, SIVsyk, SIVsm, SIVagm, and SIVl'hoest. SIVrcm and SIVdrl are not shown since sequence analyses of their complete genomes were not available. Horizontal branch lengths indicate the degree of divergence as compared to the scale at the bottom of the figure (0.1 amino acid replacement per site). Asterisks indicate that the clade to the right was found in 100% of the bootstrap values of the neighbor-joining analysis.

from sooty mangabeys to humans (Gao *et al.*, 1991). More recently, a similar relationship has been described between SIV isolates from chimpanzees (SIVcpz; *Pan troglodytes*) and HIV-1, consistent with the origins of the HIV-1 epidemic in chimpanzees (Huet *et al.*, 1990; Janssens *et al.*, 1994; Gao *et al.*, 1999).

Given the genetic relatedness of the immunodeficiency viruses of nonhuman primates with the etiologic agents of the human acquired immunodeficiency syndrome (AIDS), the human immunodeficiency viruses (HIV-1 and HIV-2), it is perhaps not surprising that these viruses share many biological properties. Indeed, much of the interest in these viruses stems from their similarities to HIV-1 and HIV-2 in genetic structure, gene regulation, tropism, and cellular receptor usage. SIV and HIV share tropism for CD4+ T lymphocytes and macrophages, and utilize CD4 as well as the chemokine receptor molecule, CCR5, for viral entry. The vast majority of SIV isolates do not use the CXCR4 chemokine receptor molecule for entry which is one characteristic that distiguishes them from HIV-1 (Unutmaz *et al.*, 1998). As shown in Fig. 2, the genetic organization of SIV and HIV are similar. The basic genome structure of the majority of the primate lentiviruses represented in SIVagm, SIVmnd, SIVsyk, SIVsun, and SIVlhoest is *gag-pol-vif-vpr-tat-rev-env-nef*. SIVsm and HIV-2 share a common novel gene, *vpx*, in the central region of their genomes, and SIVcpz and HIV-1 share the *vpu* gene. The major utility of SIV as an animal model for AIDS arises from the observations that many SIV isolates can infect Asian macaques (*Macaca sp.*) and induce an AIDS-like syndrome similar to HIV infection of humans,

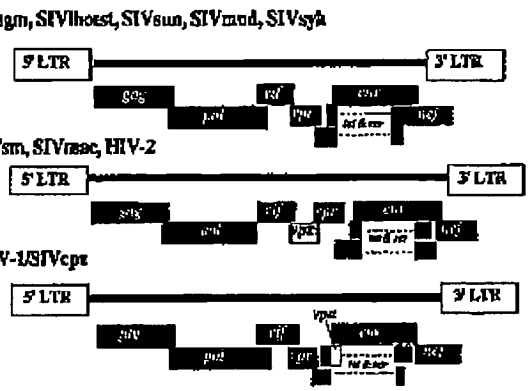

FIGURE 2 Genomic organization of SIV. A schematic representation of the genome structure of various SIV and HIV strains is shown. (A) The majority of SIV strains have a structure as depicted (*gag-pol-vif-vpr-tat-rev-env-nef*) where each gene is represented by a black rectangle. (B) SIVsm and HIV-2 have an additional gene, Vpx, shown by the white rectangle and (C) SIVcpz and HIV-1 have an additional gene, Vpu, shown by the white rectangle.

as reviewed in Allan (1991), Hirsch and Johnson (1994), and Letvin and King (1990).

II. SIV as a Model for Human AIDS

A. Natural Infection

Although SIV infection appears to be highly prevalent among free-living African primates, there is no evidence that infection is associated with any adverse consequences. The best evidence for the apathogenic nature of SIV infection in African primates comes from studies of sooty mangabeys housed in North American primate centers. Although up to 90% seropositivity has been reported in these colonies, there is no evidence of AIDS in observation over the entire life span of these animals. The lack of disease association provides a model to study successful host mechanisms in dealing with lentiviral infection. Unfortunately, there are few of such animals in captivity, their immunology is poorly characterized, and the viruses infecting such animals are genetically diverse. Therefore experimental models of natural infection can provide a system for examining the host mechanisms responsible for protecting against development of AIDS.

One such model is experimental SIVagm infection of African green monkeys (AGM), which also does not result in disease development in AGM. This model becomes more interesting when one realizes that experimental transfer of SIVagm from a naturally infected AGM to one of the Asian macaque species frequently results in an AIDS-like syndrome with remarkable similarities to human AIDS (Hirsch *et al.*, 1995). Thus SIV strains are not attenuated per se; rather it is the unique virus–host interaction in African monkeys that results in lack of disease. At the present time the host mechanism(s) responsible for the lack of virulence of these viruses in their natural host species have not been delineated.

B. Pathogenic Experimental Infection

The observation that SIV induces AIDS in macaques actually came about by serendipity. In the 1980s, an unusual clustering of lymphomas and immunodeficiency-associated disorders was noted in a colony of captive macaques at the New England Regional Primate Research Center. These observations eventually led to the isolation of simian immunodeficiency virus (SIV), which was designated SIVmac to indicate its apparent origin in macaques (Daniel *et al.*, 1985; Letvin *et al.*, 1985). Additional related SIV isolates were identified in stumptailed macaques (SIVstm) at the California Regional Primate Center and a pigtailed macaque from the Washington Regional Primate Research Center (SIVmne; Benveniste *et al.*, 1986). In

parallel, investigators at the Tulane primate center, conducting leprosy studies in sooty mangabeys, observed that transplantation of tissues from a sooty mangabey to a rhesus macaque resulted in AIDS (Murphey-Corb *et al.*, 1986). Investigators at the Yerkes primate Center also identified SIV in their colony of sooty mangabey monkeys (Fultz *et al.*, 1986) and the viruses from these two centers were designated SIVsm. After molecular characterization of SIVmac and SIVsm, it became apparent that these were highly related viruses (Hirsch *et al.*, 1989). Based upon the presence of SIVsm in feral populations of sooty mangabeys in West Africa (Marx *et al.*, 1991) and sequence analysis of these and North American isolates of SIVsm/SIVmac (Chakrabarti *et al.*, 1987; Hirsch *et al.*, 1989), researchers have concluded that SIVmac, SIVstm, and SIVmne are actually inadvertant transmissions of SIVsm into macaque populations through housing with sooty mangabeys in captivity (Hirsch and Johnson, 1994; Sharp *et al.*, 1995).

Many of the SIVsm and SIVmac isolates that have been studied for pathogenesis in primates are described in Table II. Other SIV isolates from each of the primate lentivirus lineages have been characterized for their pathogenic effects in their natural host species or macaques (reviewed in Allan *et al.*, 1991; Johnson and Hirsch, 1994), as summarized in Table III. Both SIVsm and SIVagm (Hirsch *et al.*, 1995) can induce AIDS in experimentally inoculated macaques. Some of these viruses do not appear

TABLE II Genetic and Pathogenic Diversity of SIV Strains

Subtype	*Strain*	*Isolate form*	*Disease potential*
SIVmac	SIVmac251	SIVmac*251*, uncloned	High, AIDS
		SIVmac*32H*, uncloned	Moderate, AIDS
		SIVmac*1*5, molecular clone	Low, AIDS
		SIVmac*BK28*, molecular clone	Low, AIDS
		SIVmac*1A11*, molecular clone	Attenuated
	SIVmac239	SIVmac*239*, molecular clone	High, AIDS
	SIVmne	SIVmne, uncloned	Moderate, AIDS
		SIVmne*E11S*, biological clone	Low, AIDS
		SIVmne*c18*, molecular clone	Low, AIDS
SIVsm	SIVsmB670	SIVsmB670, uncloned	High, AIDS
	SIVsmF236	SIVsmF236, uncloned	Moderate
		SIVsmH-4, molecular clone	Low, AIDS
		SIVsmH-3, molecular clone	Low, AIDS
	SIVsmE660	SIVsmE660, uncloned	High, AIDS
	SIVsmE543	SIVsmE543, uncloned	High, AIDS
		SIVsmE543-3, molecular clone	High, AIDS
	SIVsmm9	SIVsmm9, uncloned	Moderate, AIDS
	SIVsmPBj	SIVsmPBj14, biologically cloned	High, Acute disease
		SIVsmPBj6.6, molecular clone	High, Acute Disease

TABLE III Pathogenesis of Other SIV Lineages

Lineage	*Isolate*	*Pathogenesis*
SIVagm	SIVagm/ver-3	No disease (Cyn, Pt, AGM)
	SIVagm/verTyo1	No disease (Cyn, AGM)
	SIVagm/ver155	No disease (Pt, Rh, AGM)
	SIVagm/ver90	AIDS (PT); No disease (Rh)
	SIVagm/ver9063	AIDS (PT); No disease (Rh, AGM)
	SIVagm/gri-1	n.t.
	SIVagm/sab	No disease (Rh)
	SIVagm/tan	No disease (Cyn, AGM)
SIVsyk	SIVsyk173	No disease (Rh, Pt, Cyn)
SIVmnd	SIVmnd/GB-1	n.t.
SIVlhoest	SIVlhoest-7	AIDS (Pt)

Abbreviations: Cyn, cynomolugus macaque; Pt, pigtailed macaque; AGM, African green monkey; Rh, rhesus macaque; n.t., not tested.

to be pathogenic. For example, SIVsyk infects various macaque species but does not appear to result in AIDS in these animals. In contrast, SIVagm can produce AIDS in pigtailed macaques (but not rhesus macaques or the natural host, AGM; Hirsch *et al.*, 1995). In addition, SIVlhoest from a l'hoest monkey induces characteristic CD4 depletion in pigtailed macaques, also consistent with virulence in this species (Hirsch *et al.*, 1999).

The majority of studies have focused on the SIVsm and SIVmac viruses (reviewed in Letvin and King, 1990) since these viruses were the first to be demonstrated to induce an immunodeficiency syndrome in macaques. Both SIVsm and SIVmac cause a fatal immunodeficiency in a variety of species of macaque monkeys with an accompanying depletion of circulating CD4 lymphocytes and the onset of opportunistic infections and virally induced meningoencephalitis. The resulting disease is remarkably similar in pathology and apparent pathogenesis to human AIDS. However, in contrast to human HIV infection, where progression from initial infection to AIDS may take more than a decade, in many SIV infected macaque models these events are compressed into a 1- to 2-year period and thus into the realm of experimental feasibility.

1. Phases of SIV Infection

Similar to the human disease, experimental infection of macaques with SIVsm/SIVmac can be divided into three distinct phases, the primary infection, an asymptomatic phase, and a late phase, termed AIDS. Primary infection occurs within the first 3 weeks after intravenous or mucosal inoculation and is characterized by massive viremia; a transient leukopenia; and clinical signs such as fever, lymphadenopathy, diarrhea, rash, anorexia, and general malaise (Letvin and King 1990). SIV-specific antibodies and cytotoxic T

lymphocytes (CTL) develop (Kuroda *et al.*, 1998) and the plasma viremia resolves as the animal enters a clinically asymptomatic phase. The animals remain in an apparently healthy state although many exhibit significant lymphadenopathy and continual but gradual decline in the absolute circulating CD4 lymphocytes, along with evidence of immune activation. The sequential progression of the lymph node pathology ranges from early lymphoid hyperplasia, to dissolution of germinal centers, eventually leading to severe follicular and paracortical lymphoid depletion during the late stages of the disease (Hirsch *et al.*, 1991). The final phase, AIDS, is characterized primarily by severe depletion of CD4 lymphocytes and the onset of opportunistic infections such as cytomegalovirus (CMV), *Pneumocystis carnii* pneumonia, and mycobacterial infections (Baskin *et al.*, 1988; Hirsch and Johnson, 1994; McClure *et al.*, 1989; Zhang *et al.*, 1988). In HIV-1 infected humans, a switch in the major coreceptor (from CCR5 to CXCR4) used by HIV-1 has been observed in about 50% of patients as they progress to AIDS. Coreceptor use of SIV and HIV is reviewed in Unutmaz *et al.* (1998). The coincidence of this switch has lead some to postulate that the increased cytopathic effects of these viruses (due to the coreceptor switch) is associated with an increase in virulence of late-stage isolates (reviewed by Fenyo *et al.*, 1989). These theories are impossible to address with HIV in humans. Recent studies with the SIVmne/macaque model demonstrate that viruses that evolve in an infected macaque become more virulent as assessed by increased rapidity in AIDS induction in naive macaques (Edmondson *et al.*, 1998; Kimata *et al.*, 1999). Interestingly, the increase in virulence of SIVmne is not associated with a change in coreceptor use (Kimata *et al.*, 1999).

2. AIDS-Inducing Strains of SIV

As is evident from Table II, the pathogenicity of SIVsm/mac strains varies significantly from attenuated to highly pathogenic. A wide range of isolates are available as uncloned virus stocks along with numerous molecularly or biologically cloned viruses. The clinical course of infected animals varies significantly based on the virus strain and the individual animals' response to infection. Some of these factors that can influence the pathogenicity of SIV isolates are the source of the virus, strain of virus, whether it is molecularly cloned, the species of animal inoculated, and the tissue culture passage history of the virus. Some strains are relatively nonpathogenic, others result in AIDS after a long latency, and some induce AIDS more rapidly.

This situation affords a spectrum of experimental options that is broad enough to be confusing. However, it also means that virtually regardless of the specific aspect of HIV pathogenesis of interest, there is an SIV infection model that nicely recapitulates the essential aspects of the process. Thus, uncloned SIVmac251 (Letvin *et al.*, 1985), SIVsmB670 (Zhang *et al.*, 1988), SIVsmE660, and SIVsmE543 (Hirsch and Johnson, 1994) are all highly pathogenic isolates. Characteristically, 10–30% of animals inoculated with

such strains fail to develop SIV-specific immune responses and die rapidly within 6 months of inoculation, as depicted in a Kaplan–Meier plot of survival of SIV-infected macaques in Fig. 3. It is fairly rare to observe long-term survivors (long-term nonprogressors) of infection with such highly pathogenic strains. In contrast, there are other AIDS-inducing strains that are slightly less pathogenic which do not induce rapidly progressive disease. Such strains include SIVmne (Benveniste *et al.*, 1988), SIVsmF236 (Zhang *et al.*, 1988; Hirsch and Johnson, 1994), and SIVsmm9 (McClure *et al.*, 1989). In general, all of the uncloned SIVsm/SIVmac isolates exhibit some virulence. There are fewer examples of pathogenic molecularly cloned (or biologically cloned) SIV isolates. Most of the molecularly cloned viruses are minimally, if at all, pathogenic. These viruses include SIVmac1A11 (Luciw *et al.*, 1992; Marthas *et al.*, 1993), SIVsmH-4 (Hirsch *et al.*, 1989), SIVmacBK-28 (Edmondson *et al.*, 1998), and SIVsm62d (Hirsch *et al.*, 1998a). There are only a handful of AIDS-inducing molecularly cloned SIVs, including SIVmac239 (Kestler *et al.*, 1988) and SIVsmE543-3 (Hirsch *et al.*, 1998).

3. Strains with Variant Pathogenesis

Only one strain of SIV (SIVsm/PBj) appears to be acutely lethal. Experimental infection of pigtail macaques results in a highly reproducible syn-

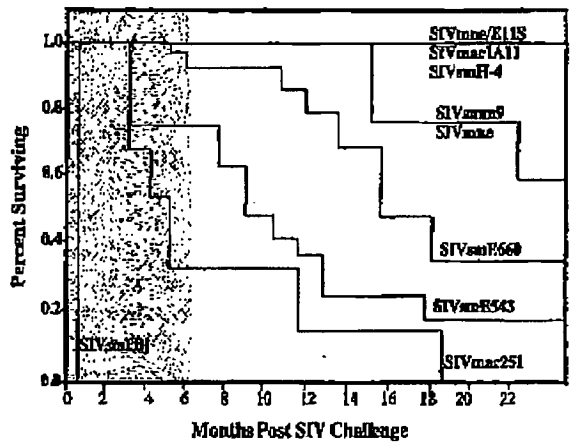

FIGURE 3 Variability in survival of macaques infected with various strains of SIVsm and SIVmac as depicted in a Kaplan–Meier plot. Some strains such as SIVmac251, SIVsmE660, and SIVsmE543 are highly pathogenic, whereas other strains are minimally pathogenic. Characteristically, 10 to 30% of macaques inoculated with highly pathogenic strains develop AIDS within 6 months of inoculation. These plots were constructed from data on survival of macaques inoculated with different SIV strains (Baskin *et al.*, 1988; McClure *et al.*, 1989; Hirsch and Johnson, 1994; Letvin *et al.*, 1985; Letvin and King, 1990).

drome of severe diarrhea and death by 7 to 14 days postinoculation (Foltz *et al.*, 1989; Fultz and Zack, 1990; Dewhurst *et al.*, 1990; Novembre *et al.*, 1993). Originally isolated from a pigtailed macaques inoculated with the AIDS-inducing SIVsmm9 strain, SIVsmPBj is an interesting virus characterized by distinct *in vitro* properties and *in vivo* pathogenesis. *In vitro*, in contrast to other SIV and HIV isolates, the virus replicates efficiently in cultures of resting T lymphocytes. At least part of this phenotype appears to be related to a characteristic mutation in the *nef* gene that introduces an ITAM motif associated with the ability to activate T cells. Introduction of this mutation into the *nef* gene of the AIDS-inducing SIVmac239 strain partially recapitulates the PBj phenotype (Du *et al.*, 1995). *In vivo* infection with the PBj virus is characterized by early high-level viral replication in the lymphoid tissues of the gastrointestinal tract. This is accompanied by a massive infiltration of lymphocytes and inflammatory changes and production of IL-6 and other cytokines, leading to diarrhea, dehydration, erosion of the mucosa, and death within 2 weeks following inoculation (Fultz and Zack, 1994).

In addition to the SIV viruses described above which cause a gradual depletion of CD4 cells that mimics the pattern seen in human infection with HIV-1, experimental infection systems have been described that result in dramatic, rapid, and virtually complete loss of CD4 cells. One such system involves infection of pigtailed macaques with the HIV-2 isolate HIV-2/287 (Hu *et al.*, 1993; Watson *et al.*, 1997a). This particular isolate was derived from the HIV-2/EHO isolate by serial passage in pigtailed macaques through which the virus acquired virulence. Following inoculation, high levels of viral replication are observed, while circulating CD4 cells decline to virtually unmeasurable levels over a period of weeks. Histopathologic analyses demonstrate extensive T-cell depletion of lymphoid tissues and confirm that the loss of measurable circulating cells is due to true loss of the cells.

A similar pattern of pathogenesis is seen with certain engineered viruses, designated SHIVs, for simian/human immunodeficiency viruses. Engineered by recombinant techniques for use in vaccine experiments in which investigators wished to study the envelope glycoprotein of HIV-1 in an *in vivo* nonhuman primate model, the SHIVs are chimeric viruses that essentially consist of viral cores composed of SIV internal structural proteins surrounded by HIV-1 envelopes. The subtleties of the exact construction of different SHIVs, including the source of accessory genes and regulatory sequences (HIV or SIV), are reviewed in Lu *et al.* (1996). The initial SHIVs that were inoculated into animals proved to replicate only transiently at low levels and were aparhogenic (Li *et al.*, 1992; Shibata *et al.*, 1991). It was only after varying degrees of *in vivo* passage that pathogenic SHIVs were successfully isolated (Joag *et al.*, 1997; Lu *et al.*, 1998; Shibata *et al.*, 1997). Thus there are a number of independently isolated pathogenic SHIVs. SHIV/89.6P was derived from SHIV89.6, which expressed the primary isolate 89.6 envelope. SHIV/KU-1 (Joag *et al.*, 1997) was derived from a SHIV expressing the HIV-1/IIIB envelope (Li *et al.*, 1992), and SHIV/DH12R was derived from SHIV expressing the envelope of the primary HIV-1 isolate, DH12 (Shibata *et al.*, 1997). These viruses are associated with sustained, high-level viral replication (similar to levels seen with pathogenic SIV isolates) and rapid, virtually complete depletion of CD4 cells, strongly reminiscent of the pattern seen in HIV-2/287 infection of pigtail macaques. Each of the SHIV strains (SHIV89.6P, SHIV/KU-1 and SHIV/KU-2, and SHIV/DH12R) that acquired virulence express a CXCR4-utilizing envelope. Interestingly a number of mutations in the envelope gene acquired through the course of *in vivo* passage appear to be conserved between different SHIV isolates that became pathogenic through *in vivo* passage. Strains which express a CCR5-utilizing envelope do not appear to induce the rapid peripheral CD4 lymphocyte depletion (Harouse *et al.*, 1999). These viruses provide a system for the assessment of candidate vaccines that incorporate HIV-1 envelope glycoprotein as a part of the immunogen with a rigorous pathogenic challenge, allowing evaluation of both laboratory and clinical endpoints (Lu *et al.*, 1996).

4. *Undefined Host Factors Influence Variable Disease Outcome*

For a given virus, there is generally a characteristic associated range of pathogenicity in a given macaque species that is broadly consistent from experiment to experiment and correlates with the extent of viral replication. Interestingly, even a given virus can vary in replicative capacity and pathogenicity *in vivo* in different macaque species. The biologically cloned virus SIVmne/E11S (Benveniste *et al.*, 1994) and the isolate from which it was derived (SIVMne; Benveniste *et al.*, 1986) exhibit among the clearer examples of species-dependent pathogenicity upon experimental infection. *Macaca mulatta* can be infected, but appears to be relatively resistant to pathogenic consequences of infection. In contrast, infection is associated with depletion of CD4+ T cells and development of AIDS over 1–2 years in the majority of inoculated *Macaca nemestrina*, while experimental inoculation of *Macaca fasicularis* results in AIDS at a slightly lower frequency and typically after a longer duration. The underlying basis for this phenomenon is not well understood. These differences in pathogenicity in different species correlate broadly with the extent of replication by the different viruses in different species. The species of macaque used for experimental infection also has a major impact upon pathogenesis. In general, pigtailed macaques (*M. nemestrina*) appear to be the most susceptible to the majority of SIVs and can even be infected with strains of HIV-1 (albeit with very low viral replication levels) (Agy *et al.*, 1992). Thus SIVagm and SIVsmPBj are uniformly pathogenic in this species but not in rhesus macaques (Hirsch *et al.*, 1995; Lewis *et al.*, 1992). However, there are some exceptions to this rule.

For example, SIVmac239 is highly adapted to rhesus macaques and is significantly less pathogenic in pigtailed and cynomologus macaques.

Regardless of the relative virulence of a particular SIV isolate, considerable biologic variation can occur between different identically inoculated animals. Thus, the disease course in an individual animal can vary from rapid to intermediate to slow. There is a spectrum of potential responses to SIV infection with specific strains and the spectrum varies for each individual isolate. A small number of animals inoculated with a highly pathogenic strain of SIV may not develop SIV-specific antibody and will die rapidly, whereas others mount a more effective immune response and survive for longer periods of time (Zhang *et al.*, 1988). The specific host factors responsible for this great variation in response to infection are not known. The disease course in rhesus macaques can be predicted to some degree by assessment of *in vitro* susceptibility to SIV of PBMC from individual macaques (Lifson *et al.*, 1997).

III. Viral Load Measurements as a Prognostic Indicator

A. Measurement of Viral Load

In the course of characterizing various systems of different species of monkeys infected with different strains of SIV it has become clear that the extent of viral replication, or "viral load," is one of the most important determinants of pathogenesis (Hirsch *et al.*, 1996; Lifson *et al.*, 1997; Watson *et al.*, 1997; Staprans *et al.*, 1999). Viral load is most conveniently assessed by measurement of the level of virion-associated SIV RNA in plasma. This observation, which parallels similar observations in HIV-infected humans (Mellors *et al.*, 1996; O'Brien *et al.*, 1996), has been enabled by the development of laboratory methods that allow the sensitive, accurate, and precise quantitation of viral load in specimens from infected animals. Prior to considering in detail the role of viral load measurements in understanding SIV pathogenesis and in the evaluation of experimental vaccines and therapies, we briefly review the approaches used to perform such measurements.

1. *Classic Methods of Measuring Viral Load*

Initial approaches for measurement of viral load in SIV-infected animals used classic methods, such as limiting dilution infectivity cultures with indicator cells for plasma or PBMC, capture immunoassays for detection of viral proteins, or immunohistochemical/*in situ* hybridization analysis of tissues. However, infectivity cultures are expensive and time and labor intensive and suffer from limitations in assay reproducibility and dynamic range. Capture immunoassays for viral proteins are simple and convenient, but are of limited sensitivity and may be seriously confounded by interference from

endogenous antibodies present in specimens from infected or vaccinated animals. Analysis of tissues by immunohistochemistry for SIV antigens or *in situ* hybridization for SIV RNA are critical methods for localizing infected cells in tissues but are difficult to standardize for quantitative purposes. In addition, there is considerable variation in the expression of virus in different tissues that could be affected by sampling. Finally, obtaining the samples is relatively invasive as compared to blood sampling, even for those tissues that are most readily analyzed (tonsils and peripheral lymph nodes). This limits longitudinal assessments of viral load. As was the case for HIV, it became apparent that nucleic-acid-based approaches to assay plasma viral RNA would provide the best combination of feasibility, cost-effectiveness, and assay performance characteristics for measurements of SIV viral load.

2. *Branched DNA Methods*

As for HIV, the available nucleic acid methods for quantifying viral load can be classified as either target-amplification or signal-amplification approaches. In each instance, an amplification step is introduced to allow indirect measurement of the very small (in absolute terms) amounts of viral RNA present in test samples, amounts too small to be measured without amplification. The primary signal amplification method in use for quantification of SIV uses an approach designated "branched DNA" or bDNA detection. In this method, solid-phase bound oligonucleotides are used for sequence-specific capture of SIV RNA, which is then subsequently detected by oligonucleotides containing complementary sequences for binding to the captured SIV RNA probes. Extensive "branched DNA" arms, to which are conjugated numerous alkaline phosphatase moieties, allow sensitive and quantitative chemiluminescent detection (Marx *et al.*, 1996; Staprans *et al.*, 1999). The method is robust, with good precision, although it requires a relatively large sample volume and has not been as sensitive as other methods (see below). A newer version of the basic assay has improved sensitivity.

3. *Quantitative Competitive PCR Methods for Assaying Viral RNA*

Other available methods depend on amplification of the target template itself to achieve quantitation in a measurable range. The most widely used of these methods are based on variations of the polymerase chain reaction. However, as reviewed in detail elsewhere, there are serious intrinsic problems in attempting to use PCR for quantitative applications (Piatak *et al.*, 1996; Piatak and Lifson, 1997). To overcome these problems, two main approaches have been employed. In the first approach, designated internally controlled PCR, or competitive PCR, or sometimes quantitative competitive PCR (QC-PCR), a synthetic internal control template is spiked into the test sample. This internal control template is designed to use the same primers as the test target template and to be reverse transcribed and PCR amplified

with efficiency comparable to the test template, but to be independently quantifiable. The basic approach thus is based on the premise that by testing a fixed but unknown amount of test template against a limited bracketing range of spiked internal control template, the amounts of amplified product for unknown and test template at the end of PCR can be measured. Since both templates are reverse transcribed and amplified with comparable efficiency, the ratio of the measurable amounts of products following amplification should reflect the ratio of target templates prior to amplification. Based on a regression of the ratio of measured postamplification products for the two templates as a function of the input copy number of the control temples, the titration equivalence point can be determined by interpolation. The key feature of this approach is that it is based on relative quantitation of target and internal control templates, not absolute endpoint quantitation of target template. Thus it avoids many of the intrinsic problems associated with absolute quantitation of endpoint PCR amplifications (Piatak *et al.*, 1993, 1996; Piatak and Lifson, 1997).

4. *Real-Time Methods for Assaying Viral RNA*

Internally controlled PCR/RT–PCR approaches have proved to be sensitive, robust, and reliable and have been used extensively in pathogenesis studies and in the evaluation of experimental vaccines and therapies in various SIV model systems (Hirsch *et al.*, 1996, 1998; Nowak *et al.*, 1997; Lifson *et al.*, 1997; Tsai *et al.*, 1998, Van Rompay *et al.*, 1998). However, these approaches are time and labor intensive, which limits throughput. Recently, new approaches have been developed based on kinetic PCR or "real-time" PCR (Heid *et al.*, 1996; Gibson *et al.*, 1996; Livak *et al.*, 1995; Suryanarayana *et al.*, 1998). The key feature of these methods is that the measurement is based on kinetic or real-time measurements of accumulating product during ongoing PCR amplification rather than endpoint measurements of accumulated amplified PCR product at the conclusion of the reaction. This confers several advantages. First, it allows measurements to be performed during the earliest stages of the exponential phase of PCR amplification. This period exhibits the most consistent relationship between input template copy number and PCR product. Second, the kinetic nature of this approach provides an extremely broad linear dynamic range. Finally, since measurements are obtained during the PCR amplification, there is no need for separate analysis of amplified products at the conclusion of amplification. This factor increases throughput and minimizes potential for PCR back-contamination associated with manipulation of amplified material.

To realize these advantages requires the ability to sequentially and noninvasively monitor accumulation of specific amplicons during ongoing PCR reactions. This technical challenge has been elegantly solved with instrumentation that provides for light excitation of ongoing PCR reactions and quantitative collection of the resulting emitted fluorescence signal. The fluorescence signal is derived via a variety of reagents and schemes that depend on release from fluorescence resonance energy transfer-mediated quenching of fluorescence from fluorochrome-labeled hybridizing oligonucleotide primers or probes. Release from quenching is obligately and quantitatively linked in a proportional manner to specific amplification of the target sequence. While a detailed description of these methods is beyond the scope of this chapter, they provide excellent sensitivity, precision, and dynamic range, with excellent specimen throughput.

Real-time PCR methods represent a significant advance in these regards, although it is important to note that there can be pitfalls with this emerging technology. The potentially severe errors in quantitation can be introduced by sequence mismatches between probes and cognate target sequences to which they are intended to hybridize (Suryanarayana *et al.*, 1998). In addition to PCR-based methods, there are a number of other technologies based on cyclical enzymatic target-amplification strategies. Discussion of these less widely used methods is beyond the scope of this chapter.

B. Viral Replication in Experimental Lentiviral Infection of Primates

Measurements of viral load through quantitation of virion associated viral RNA in plasma has been invaluable in defining the relationship between viral replication patterns and pathogenesis in different SIV infection models. As noted above, there are a number of different systems used in experimental infection studies, varying in both the species of macaque employed and the strains of virus used. Indeed, one of the principal advantages of the SIV-infected macaque as an animal model system for AIDS is the ability to define the amount, route, dose, and timing of inoculation and the identity of the inoculating virus.

1. *SIV Infection of Adapted Natural Host Species*

As described above for the families of SIVs, there appear to be natural host species in which the virus does not appear to be pathogenic. Given the well-established relationship between level of viral replication and pathogenicity, one obvious hypothesis is that the adapted host species have simply developed mechanisms to limit viral replication, thereby preventing pathogenicity. However, available studies of the natural host for SIVsm, sooty mangabeys, suggest that the levels of viral replication are comparable to the levels seen when the same viruses are used to infect new host species, such as rhesus or pigtail macaques (Rey-Cuille *et al.*, 1997; Knut *et al.*, 1998, Villinger *et al.*, 1996, 1999). The high viral load in such animals is confirmed and supported by the ease with which virus can be isolated from plasma of such animals as well as by *in situ* hybridization of lymphoid tissues of sooty mangabeys. Additional studies aimed at determining the basis of nonpatho-

genicity of these viruses in the adapted host will hopefully identify the responsible host factors. Progress in this area may provide important insights into understanding, and ultimately preventing, the pathogenesis of AIDS.

In a similar vein, analysis of African green monkeys infected with SIVagm strains is also of interest. The technical issues involved in plasma viral load measurement in African green monkeys are more complicated due to the greater genetic diversity among SIVs infecting these species. As a result, there is not a good consensus of understanding within the field concerning quantitation of plasma viral load levels in various different African green monkey species naturally or experimentally infected with different SIVagm isolates. Studies of tissues from naturally infected African green monkeys revealed that the majority have very low expression of virus in tissues (Beer *et al.*, 1996; V. Hirsch, unpublished observations). Real-time assays for viral RNA levels in plasma will be necessary to address whether the viral load in such animals is lower than that observed in sooty mangabeys. This will require the development of species-specific primers and probes to reliably detect the four specific SIVagm subtypes within African green monkeys (SIVagm/tan, SIVagm/ver, SIVagm/gri, and SIVagm/sab). However, once these problems are resolved, additional studies to characterize the rate and extent of viral replication, and the basis of nonpathogenicity in this adapted host species, will also be of great interest.

2. *Experimental Pathogenic Infection of Macaques*

There are a number of other experimental systems characterized by much greater pathogenicity, including infection of rhesus or pigtail macaques with SIV viruses such as SIVsmE660 (a biological swarm), SIVsmE543-3, SIVmac251, SIVmac239, or SIVsmB670. Infection with these viruses generally follows a consistent pattern, illustrated for SIVsmE660 in pigtail macaques in Fig. 4. Following intravenous inoculation, virus is first detectable in plasma within 3 to 7 days postinoculation. Plasma SIV RNA levels increase exponentially, reaching peak values from 10 to 20 days postinoculation. Over the next couple of weeks, there is typically a down-modulation of circulating virus levels, of varying degrees, leading in most animals to a relative stabilization of plasma viral load at what has been termed the postacute viral load "set point" or "inflection point," approximately 6–8 weeks postinoculation. Plasma viral load at this "set point" is broadly predictive of the subsequent clinical course, with animals that show higher viral loads at this time showing persistently elevated plasma virus levels and on average a more rapid progression to AIDS and death (Hirsch *et al.*, 1996; Watson *et al.*, 1997). Conversely, the small percentage of animals that show lower levels of plasma SIV over this time interval show persistently restricted viral load and much slower disease progression. In some instances, these animals exhibit a nonprogressive clinical course, with low or unmeasurable levels of plasma virus, implying host control of readily demonstrable persistent infection. This situation mimics the rare human patients with long-term

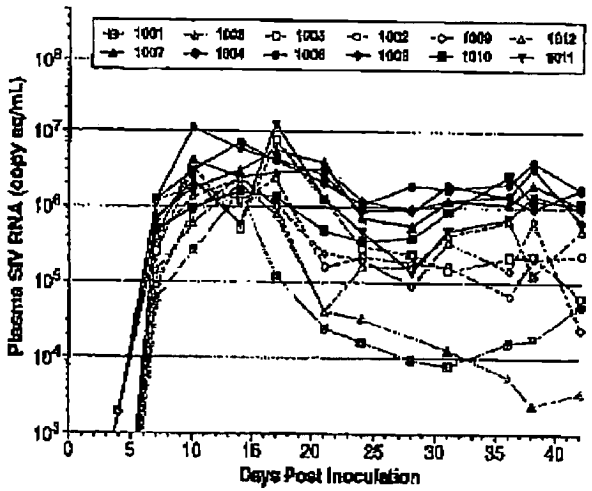

FIGURE 4 Variability in plasma viremia within a strain in a cohort of pigtailed macaques is shown over the first 40 days after intravenous inoculation of SIVsmE660 (Lifson *et al.*, 1997). Macaques with rapidly progressive infection that failed to seroconvert are shown in black symbols with solid lines. Those macaques with partial control of viremia are shown with open symbols and dashed lines and those which controlled viremia to a significant extent (<10^6/ml) are shown with shaded symbols.

nonprogressive HIV-1 infection (Pantaleo *et al.*, 1995; Cao *et al.*, 1995). A significant percentage of animals show little or no evidence of control of viral replication, as measured by plasma viremia. These animals typically fail to seroconvert and experience a rapidly fatal clinical course, generally dying less than 6 months postinoculation with massive levels of plasma viremia (up to 10^9 copies/ml). As shown in Fig. 5, there is a remarkable correlation between relative viral load assessed by *in situ* hybridization and that assessed by plasma viral RNA assays. Thus plasma viral load measurements appear to reflect the ongoing virus expression in SIV-infected macaques (Lifson *et al.*, 1997).

Of course, the patterns described above are generalizations, and the behavior of individual animals may vary, at least in part due to the ongoing dynamics of viral evolution within the host and the influence of the host immune response. Thus, the inoculated virus can evolve through *in vivo* replication (Edmondson *et al.*, 1998; Kimata *et al.*, 1999), in some instances increasing in pathogenicity such that virus recovered from late in infection may show greater and more rapid pathogenicity on inoculation into new naïve hosts (Kimata *et al.*, 1999).

3. *Minimally Pathogenic SIV Isolates*

a. Spontaneous Attenuated SIV Variants The SIV strains described above are particularly useful for studies of comparatively rapid pathogenesis or

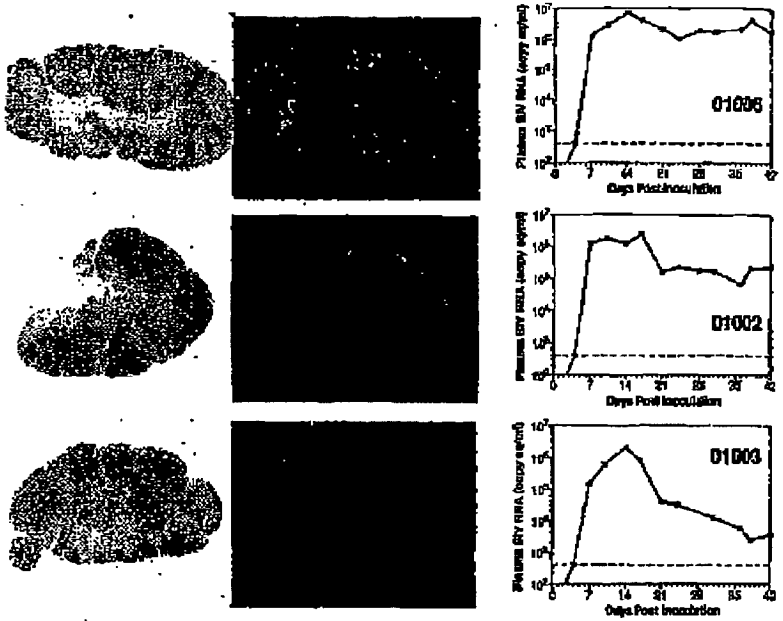

FIGURE 5 Correlation between the pattern of viral replication in three macaques inoculated with SIVsmE660 is shown on the right with the detection limit of the assay shown by a dotted line. The middle panel shows SIV-specific *in situ* hybridization of lymph node biopsies obtained at 4 weeks postinoculation and the left panel shows the corresponding H&E-stained histopathologic sections. The macaque at the top (01006) demonstrated uncontrolled viremia and progressed rapidly to AIDS with high lymph node expression of virus. The macaque in the middle panel (01002) seroconverted and decreased viremia to 100,000 copies/mL. The virus expression in the lymph node is moderate and there is evidence of trapping of virus–immune complexes on follicular dendritic cells. The macaque at the bottom (01003) controlled viremia to a greater degree to approximately 1000 copies/ml and few SIV-expressing cells were observed by *in situ* hybridization.

rigorous testing of vaccines or treatment approaches. However, there are other viruses that are essentially apathogenic, even when experimentally inoculated into host species readily susceptible to highly pathogenic infection with closely related viruses. Examples include the 1A11 molecular clone, derived from the highly pathogenic SIVmac251 swarm (Marthas *et al.*, 1989, 1993). This virus represents an interesting model in that it frequently mediates an "abortive" infection, especially after vaginal inoculation, characterized by a transient low-level viremia. The virus replicates to low levels and then becomes undetectable, while the animals remain clinically well, with no evidence of progressive SIV disease. This pattern has some similarities to another phenomenon, in which the SIVmac251 swarm, which generally replicates to high levels and is strongly pathogenic following intravenous

inoculation, can be associated with a different profile following low-dose vaginal inoculation (McChesney *et al.*, 1998). In this latter instance, the majority of animals show typical productive infection and disease course; however, a small percentage of vaginally inoculated animals show a pattern of transient low-level viremia and limited, atypical immune responses (often with variable low-level cellular responses in the absence of seroconversion). Exhaustive efforts at necropsy several years postinoculation demonstrated the persistence of virus, including replication competent virus in many instances, in most animals. However, in the vast majority of macaques that show this transient viremia pattern the infection appears to be largely latent, both virologically and clinically (McChesney *et al.*, 1998). Other examples of minimally pathogenic but ultimately AIDS-inducing strains include molecularly cloned SIVsmH-4 (Johnson *et al.*, 1990), SIVmac/BK28 (Edmondson *et al.*, 1998), and SIVsm62d (Hirsch *et al.*, 1998). Characteristically, these attenuated and minimally pathogenic SIV strains exhibit low levels of both primary and chronic plasma viremia as demonstrated by the comparison of plasma viremia in macaques inoculated with SIVsmE660 and SIVsm62d in Figs. 6A and 6B. As evident in this figure, range in both primary and postacute plasma viremia is much lower in macaques infected with the less pathogenic strain, SIVsm62d, as compared to SIVsm62d-infected macaques, which maintain lower viremia and do not show disease progression, whereas macaques at the top of the spectrum of viremia levels develop AIDS.

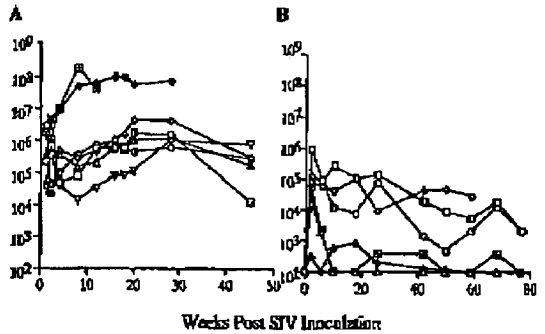

FIGURE 6 Differences in the range of plasma viremia observed in macaques inoculated with a highly pathogenic strain (SIVsmE660) and a minimally pathogenic AIDS-inducing strain (SIVsm62d) are shown. (A) The range of viremia in SIVsmE660-inoculated rhesus macaques (Hirsch *et al.*, 1996). The two macaques with the highest viremia in A died with AIDS by 12 and 32 weeks respectively, whereas the others survived for approximately 1 year. (B) Lower plasma viremia in macaques inoculated with the less pathogenic SIVsm62d molecularly cloned virus. The two animals with higher relative viremia (open symbols) died with AIDS at 58 and 77 weeks postinoculation, whereas the other macaques remained healthy for over 3 years. These animals are described in Hirsch *et al.* (1998a).

b. Genetically Modified Attenuated SIV Variants An additional example of the relationship between viral replication levels and pathogenicity can be drawn from studies of attenuated strains of SIV generated by mutational deletion of accessory genes in an effort to develop strains suitable for evaluation as candidate live attenuated vaccine strains. A series of deletion mutants have been developed and evaluated. The wild-type virus from which the deleted mutants were constructed, SIVmac239, establishes a high-level persistent viremia and pathogenic, progressive infection in the vast majority of inoculated rhesus macaques (Kestler *et al.*, 1988). In striking contrast, in the majority of inoculated animals the deleted mutants show a blunted peak viremia that resolves, with plasma SIV RNA levels generally decreasing to below the level of detection in most assays (Desrosiers *et al.*, 1998). The degree of blunting of the peak in *in vivo* viremia correlates with the extent of attenuation through mutation (Desrosiers *et al.*, 1998; Johnson *et al.*, 1999).

4. Viral Replication Patterns of Viruses with Variant Pathogenesis

In addition to the experimental SIV infection systems described above, there are some other systems involving experimental infection of macaques that result in variant patterns of pathogenesis rather than the typical progressive infection leading to AIDS. These systems have been effectively used for specific experimental purposes.

a. SIVsmPBj The kinetics of viremia in SIVsmPBj-inoculated macaques is more rapid than observed with AIDS-inducing strains of SIV, frequently peaking by 7 days after intravenous inoculation (Hirsch *et al.*, 1998; O'Neil *et al.*, 1999) versus 11 to 14 days for other SIVmac and SIVsm strains (Lifson *et al.*, 1997). However, the actual peak levels are not significantly higher than that observed with AIDS-inducing strains. Plasma viremia is accompanied by a decline in all lymphocyte subsets, as illustrated in Fig. 7A. The kinetics of viremia in intrarectally inoculated macaques is delayed by 3 to 4 days as compared to those inoculated intravenously (Fig. 7B). In keeping with the relationship between viral replication levels and pathogenesis seen in other systems, site-directed mutants of SIVsmPBj (nef, vpr, or vpx mutants) that show blunted *in vivo* viral replication also show markedly blunted pathogenesis, with animals surviving infection (Novembre *et al.*, 1997; Hirsch *et al.*, 1998). While this pattern of pathogenesis is sufficiently different from the typical course of progressive SIV or HIV infection leading to AIDS to not be an optimal model, it has been usefully employed to study specific questions in AIDS pathogenesis (Hirsch *et al.*, 1998).

b. Acutely CD4 Depleting Viruses Overall, the kinetics of viral replication following infection with the acutely CD4-depleting SHIV isolates, as reflected by plasma SIV RNA levels, parallel the pattern seen for pathogenic

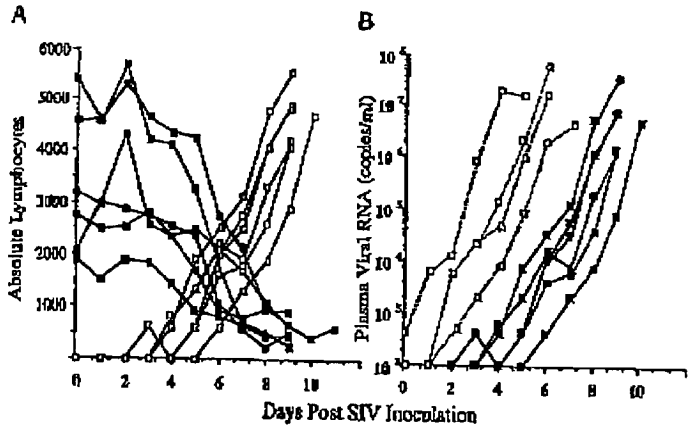

FIGURE 7 (A) The kinetics of viremia (open symbols) and accompanying lymphopenia (black symbols) in macaques inoculated with the acutely lethal, molecularly cloned, SIVsmPBj6.6 extracted from data presented in Hirsch *et al.* (1998b). (B) The delay in viremia observed in macaques inoculated intrarectally (black symbols, solid lines) as compared to those inoculated intravenously (open symbols, dotted lines).

SIV isolates (Reimann *et al.*, 1999). Infection with pathogenic SHIV isolates leads to the rapid development of high levels of plasma viremia, typically as high and as rapidly or slightly more rapidly than is observed for pathogenic SIV isolates (Lu *et al.*, 1998; Joag *et al.*, 1997, 1998; Reimann *et al.*, 1996; Shibata *et al.*, 1997). After viremia reaches a peak there can be some modest down-modulation of virus levels, but, in general, levels remain high through the period of CD4 depletion. With extensive depletion of CD4+ T cells there may be some modest decrease in levels of circulating virus, although it is interesting that moderate levels of plasma viremia are maintained even after depletion of CD4+ T cells from the circulation and lymph nodes is virtually complete.

In striking contrast, infection with the nonpathogenic SHIVs, from which the pathogenic, acutely CD4-depleting SHIVs are derived by *in vivo* passage, results in only transient viremia, reinforcing the relationship between levels of viral replication and pathogenesis *in vivo* (Reimann *et al.*, 1999; Li *et al.*, 1992; Shibata *et al.*, 1991). For the pathogenic SHIVs, a greater inherent cytopathicity for CD4+ T cells may also contribute to the dramatic CD4-depleting phenotype and overall pathogenesis *in vivo*. All of the SHIVs that induce the rapid CD4 depletion utilize CXCR4 as their coreceptor. Few SIVs that utilize CCR5 have been constructed (Luciw *et al.*, 1992; Harouse *et al.*, 1999) and these viruses do not appear to cause the acute peripheral CD4 depletion. A recent study has demonstrated, however, that a CCR5-using SHIV causes depletion of CD4+ intraepithelial lymphocytes in the gastrointestinal tract (Harouse *et al.*, 1999) similar to that seen in pathogenic SIV infection (Veazy *et al.*, 1998).

IV. Modulation of Viral Replication by Partially Protective Vaccines

Vaccination includes the prophylactic immunization as well as the immunization to modify disease during the chronic stage of infection. Therapeutic vaccination is beyond the scope of this chapter and is not discussed. Quantitative assays of viral load provide an extremely useful tool in the experimental evaluation of vaccines for AIDS. Nucleic-acid-based viral-load studies provide among the most sensitive means of evaluating protection from challenge in vaccine studies. The absence of detectable viral RNA in plasma, and viral RNA or DNA in PBMC and lymph node cells, can be used to confirm complete protection from infection ("sterilizing immunity") in macaques in which there is a failure to isolate infectious virus and lack of an anamnestic antibody response. Even in many instances where complete protection from infection was not achieved, measurement of viral load has proved extremely valuable in the evaluation of experimental vaccines in the SIV system. The use of the SIV/macaque model to evaluate AIDS vaccines has been extensively reviewed (Almond and Heeney, 1998; Hu *et al.*, 1993; Letvin, 1998; Nathanson *et al.*, 1999; Schultz and Hu, 1993; Schultz and Stott, 1994). Thus the discussion in this chapter deals primarily with studies in which protection from infection was not achieved but where measurement of viral load in the postchallenge period has demonstrated vaccination-associated reductions in viral load in vaccinated animals that did become infected relative to unvaccinated controls. In many instances, long-term follow-up has shown that substantial reduction of viral load in the immediate postchallenge period can be associated with sustained modulation of viral replication and improved clinical course relative to control animals (Hirsch *et al.*, 1996).

At the present time, a number of vaccine strategies have demonstrated complete to partial protection in primate models. To those unfamiliar with the various strains of SIV (and SHIV) used in vaccine experiments, the results of challenge experiments can be difficult to decipher and vaccine modalities almost impossible to compare. It is critical to remember that the level of protection observed *is* impacted not only by the efficacy of the vaccine but also by the genetic relatedness of the vaccine virus and the challenge virus and by the virulence of the challenge virus. Assessment of vaccine studies should also include evaluation of the neutralization phenotype since some viruses may appear to be quite similar genetically but antibodies generated to one virus may not cross-neutralize the other strain. Thus a vaccine can appear to mediate complete protection from infection if the animals are challenged with a virus identical to the vaccine virus that has low virulence (low AIDS-inducing potential and low virus loads). However, the same vaccination regimen may afford little or no protection from a more robust challenge. Therefore, in evaluating vaccine studies it is critical to understand the typical viral replication profile and clinical course associated with the individual viruses used as challenge strains in vaccine trials, as described in Section III.

A. Attenuated Live SIV Vaccines

The most effective vaccine modality still appears to be attenuated live SIV. One of the first of such attenuated live SIV mutants to be used in such a fashion is the SIV mac/1A11 virus, which is a spontaneously generated attenuated molecular clone. Prior infection of macaques with the 1A11 virus resulted in protection from AIDS when the animals were challenged with a pathogenic SIV strain (Marthas *et al.*, 1990). Later studies with genetically modified strains of SIV with deletions in the accessory genes (*nef* and *vpr*) and the LTR revealed that prior infection can provide complete protection if challenge is delayed for 6 months to a year after vaccination (Connor *et al.*, 1998). Prior infection with these viruses has resulted in some of the more impressive vaccination related protection observed to date in the SIV system (Daniel *et al.*, 1992; Desrosiers *et al.*, 1998; Johnson *et al.*, 1999). However, even with this approach, broad protection against heterologous challenges has proven difficult (Lewis *et al.*, 1999; Desrosiers *et al.*, 1999). Even in such situations, a reproducible reduction in viremia has been observed (Desrosiers *et al.*, 1998; Johnson *et al.*, 1999; Lewis *et al.*, 1999). Since the level at which plasma viremia plateaus after the primary phase of infection is an excellent prognostic indicator, significant reductions in plasma viremia in such vaccinated monkeys are associated with a long-term clinical benefit in these animals (Hirsch *et al.*, 1996; Watson *et al.*, 1997b).

The potential human use of this approach is precluded for the foreseeable future by observations that in some animals inoculated with these deleted attenuated viruses, both neonates and juveniles, pathogenic infections have been observed (Baba *et al.*, 1998, 1999; Alexander *et al.*, 1999). It is nonetheless interesting that the pathogenicity was associated with much higher levels of viral replication than is typically observed in the majority of animals receiving these viruses and with "compensatory" mutations that might be expected to increase viral replication levels (Desrosiers *et al.*, 1998). Thus even the exceptions to the rule of the behavior of these mutant attenuated viruses reinforces the rule of the relationship between the extent of viral replication and pathogenesis.

B. Live Viral Vectors

The best evidence for reduction in viremia as a consequence of prior vaccination has been observed in macaques immunized with live viral vectors that express SIV antigens. Thus, as with attenuated live vaccines, priming with vaccinia virus SIV envelope recombinants followed by a recombinant

envelope antigen boost can under ideal circumstances prevent infection. Complete protection has thus been observed in one study that used as the challenge virus a biologically cloned SIV isolate (SIVmne/E11S) that is minimally pathogenic in the species used for the trial (Hu *et al.*, 1992). In this situation, protection appears to be mediated by type-specific neutralizing antibodies that were fortunately matched to the challenge strain. However, when similarly immunized macaques were challenged with a slightly more pathogenic, and more heterogenous, isolate, uncloned SIVmne, only partial protection was observed spanning the spectrum from complete protection and transient infection to reduction in viral replication (Polacino *et al.*, 1999). The investigators have also observed a significant reduction in viremia in macaques immunized with vaccinia virus core antigen recombinants, suggesting that genes other than envelope can contribute in vaccine-mediated protective effects (Hu *et al.*, 1993). In a similar vein, immunization of macaques with vaccinia virus envelope recombinant virus and boosting with recombinant envelope did not prevent infection following challenge with the highly pathogenic SIVmac251, but modulation of viremia was observed (Ahmad *et al.*, 1994).

There are a number of poxviruses with unique properties that are available for use as vaccine vectors (reviewed in Paoletti *et al.*, 1996; Tartaglia *et al.*, 1998). This includes the conventional vaccinia viruses used for the smallpox eradication campaign [such as New York Board of Health (Wyeth) and Copenhagen strains]. The use of these viruses in populations where a fraction of the vaccinees might be immunosuppressed due to HIV-1 infection is problematic due to the risk of disseminated fatal vaccinia virus infection in such individuals. Therefore attenuated poxviruses have been developed for use as vaccine vectors (Meyer *et al.*, 1991; Paoletti *et al.*, 1996; Moss *et al.*, 1996). NYVAC and MVA (modified vaccinia virus Ankara) are attenuated vaccinia viruses (Blanchard *et al.*, 1998) that have been explored as potential AIDS vaccine vectors in primate models. NYVAC is genetically modified version of the New York Board of Health strain, whereas MVA was spontaneously generated through passage in chicken embry fibrobalsts. Both have severe host range restrictions in mammalian cells and are safe in immunosuppressed animal models. In addition, the avipoxviruses, ALVAC, and fowlpox are also attractive candidates since they would be immunogenic in vaccinia-immunized individuals (Andersson *et al.*, 1996). Protection from infection with HIV-2, which is apathogenic in macaques, has been observed in macaques immunized with poxvirus recombinants of HIV-2 (Abimiku *et al.*, 1995; Myagkikh *et al.*, 1995). Modulation of plasma viremia has been observed in macaques immunized with both NYVAC and MVA–SIV recombinant vaccines (Benson *et al.*, 1998; Hirsch *et al.*, 1997). Modulation of viral load was more pronounced in macaques challenged by the intrarectal route (Benson *et al.*, 1998). A small proportion of the vaccinees in both of these studies controlled virus replication to extremely low levels and have

become macaque equivalents of long-term nonprogressors (LTNP) (Abimiku *et al.*, 1997; Hirsch *et al.*, 1997). The effect on viremia in macaques immunized with MVA–SIV recombinants as compared to those immunized with a Wyeth–SIV recombinant or nonrecombinant vaccinia virus is illustrated in Fig. 8. Fowlpox recombinants expressing HIV-1 antigens have been used in combination with DNA priming. This approach significantly boosts CTL responses and has been shown to protect macaques against HIV-1 infection (Kent *et al.*, 1998). However, the relevance of this protection is unclear, since HIV-1 infection of macaques is highly transient in nature and therefore this constitutes the weakest of vaccine challenges.

C. Other Viral Vectors

A number of other nonpathogenic viruses under consideration and investigation as potential viral vectors for an AIDS vaccine are poliovirus replicons (Morrow *et al.*, 1999); adeno-associated virus (AAV) in the very early stages of development (Clark *et al.*, 1995); adenovirus (Robert-Guroff *et al.*, 1998); alphaviruses, including Semliki forest virus (SFV; Berglund *et al.*, 1997; Mossman *et al.*, 1996); and venezuelan equine encephalitis virus (VEE; Caley *et al.*, 1997). Adenovirus recombinants of HIV provided protection when used in a prime boost strategy in chimpanzees when the challenge strain was matched genetically to the vaccine virus and are capable of preventing infection (Robert-Guroff *et al.*, 1998). When a more rigorous

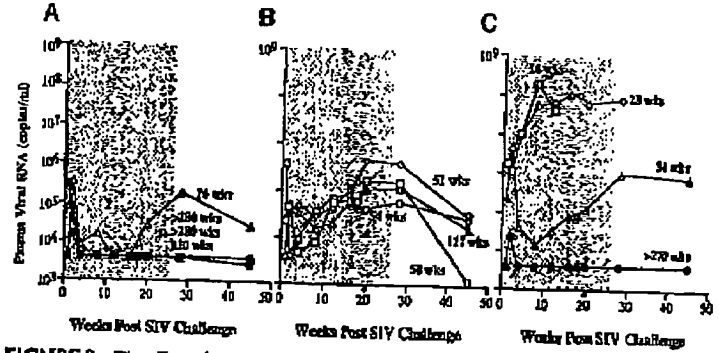

FIGURE 8 The effect of vaccination with a partially protective vaccine, MVA-expressing SIV env and gag-pol (Hirsch *et al.*, 1996). Plasma viremia during the first 50 weeks postintravenous inoculation with SIVsmE660 of macaques vaccinated with (A) MVA-expressing SIV gag-pol and env, (B) Wyeth-expressing SIV gag-pol and env, and (C) nonrecombinant vaccinia virus. The survival of the macaques in weeks is indicated by the numbers near the plot of plasma viremia. The shaded area indicates the first 6 months after challenge, the period in which rapid progressor macaques develop AIDS. Rapid progressors are plotted with open symbols, slower progressors with shaded symbols, and nonprogressors with black symbols.

challenge is used such as in the SIV/macaque model, macaques immunized with a adenovirus–SIVenv recombinant and boosted with SIVmac gp120 were not protected from infection following intravaginal challenge with pathogenic SIVmac251. However, significant reduction in plasma viremia was observed in these vaccinated macaques (Buge *et al.*, 1997). Macaques immunized with SFV-expressing envelope were protected from acutely lethal disease when challenged with SIVsmPBj, although all animals became infected. Although viremia was not characterized in these animals, it is likely that the protection observed with SFV recombinant viruses was due to blunting of acute viremia. There are no published reports on challenge studies in macaques immunized with either VEE– or AAV–SIV recombinants although these studies are in progress.

D. DNA Immunization

Another exciting strategy for generating both cellular and humoral immunity is the use of naked DNA as an immunogen as reviewed by Robinson (1997). DNA can be adminstered either intramuscularly or coated on gold particles by gene gun. Although this method is extremely immunogenic in mice, there have been difficulties in generating similar responses in primates. Nevertheless, there are some preliminary trials in which protection from infection was achieved in chimpanzees immunized with HIV-1 envelope and challenged with HIV-1/SF-2 (Boyer *et al.*, 1997). The SF-2 strain of HIV-1 is considered to be a less rigorous challenge than other HIV-1 strains such as IIIB, since it shows restricted replication in nonimmunized chimps. Others have investigated the use of HIV-1 envelope DNA in rhesus macaques. Macaques immunized with env and boosted with recombinant env were protected from infection after an intravenous challenge with SHIV/IIIB (Letvin *et al.*, 1997). As discussed above (Section II,B,3), the original parental SHIVs such as SHIV/IIIB are not pathogenic and do not replicate efficiently in macaques. Therefore, as with the experiments with chimps, this is not a rigorous challenge and will require further validation with a more robust challenge. When DNA immunization has been evaluated in the SIV/macaque model (Fuller *et al.*, 1997; Haigwood *et al.*, 1999; Lu *et al.*, 1996) reduction in viremia has been observed, consistent with a partially protective effect of this vaccine regimen. As discussed briefly above under poxvirus vectors, there is considerable promise in the approach of combining DNA immunization with a viral vector such as the attenuated poxviruses. Preliminary studies suggest that such an approach significantly boosts CTL responses (Kent *et al.*, 1998; Robinson *et al.*, 1999).

In summary, a pattern begins to emerge from evaluation of challenge results with various immunization protocols. With the exception of live attenuated SIV vaccines, the degree of protection observed in many of the trials is less than ideal. This translates into complete protection if an attenuated virus is used for challenge and significant reduction in viremia if a more robust pathogenic challenge virus is employed. However, it should also be noted that the challenges typically employed in vaccine studies in macaques are, for practical reasons, typically much more rigorous than estimates of the type of exposures involved in human infection with HIV-1. Thus, vaccine studies generally are designed using a challenge inoculum that will result in productive infection of all nonvaccinated control animals. This contrasts with estimates of infection rates for sexual transmission of HIV-1 that are generally less than 1% per exposure episode. This factor, in combination with the observation that, in general, there has been greater success in protecting against mucosal challenge than against intravenous challenge, suggests that even vaccine approaches that are less than completely protective in macaque/SIV models may still show some degree of efficacy in people. Experience with clinical studies of candidate vaccines will be required to further clarify this issue and perhaps help refine challenge models to further optimize the evaluation of vaccines in macaques.

V. Antiviral Therapy

Experimental models of SIV infection have also proven valuable in the evaluation of antiviral therapies. However, since many anti-HIV drugs are targeted to specific enzymes such as the viral reverse transcriptase and protease, and there are subtle differences in the corresponding enzymes in SIV, some anti-HIV compounds may not work as potently against SIV as they do against HIV. Nevertheless, SIV-infected macaques remain a useful model for the evaluation of compounds having good potency against SIV and HIV. In addition, they provide an extremely important model to explore questions related to pathogenesis and treatment that cannot be readily approached in HIV-1-infected human subjects, due to logistical constraints, ethical considerations, or other issues.

A. Treatment of Macaques with Antiretroviral Drugs

One compound with broad activity against many retroviruses, including SIV, is the reverse transcriptase inhibitor 9-[2-(*R*)-(phosphonomethoxy)propyl]adenine (PMPA). This compound is particularly convenient to use in SIV-infected macaques and due to its potency and pharamcokinetic profile, effective drug levels can be maintained with a single daily dose, given by subcutaneous injection, without any need to anesthetize the animals. This avoids the practical difficulties encountered in trying to achieve controlled administration of other drugs, the pharmacological or pharmacokinetic properties of which often require multiple daily doses, oral administration,

or parenteral administration via routes less convenient than subcutaneous injection. Due in part to this profile, PMPA has been used extensively in SIV studies to address a number of different, important questions.

B. Treatment of Chronic SIV Infection

PMPA has been shown to potently suppress viral replication in chronically SIV-infected macaques, although drug levels returned to essentially pretreatment baseline levels upon drug discontinuation (Nowak *et al.*, 1997; Tsai *et al.*, 1997). An example of a 14-day treatment with PMPA of three chronically SIV-infected macaques is shown in Fig. 9. Careful measurements of plasma viral load prior to, during, and upon discontinuation of drug treatment have allowed the estimation of viral dynamics parameters in SIV-infected macaques (Nowak *et al.*, 1997). These parameters are broadly comparable with the same parameters measured in HIV-infected patients treated with antiviral drugs (Ho *et al.*, 1995; Wei *et al.*, 1995), underscoring the similarity of HIV and SIV infection and reinforcing the relevance of studies in SIV-infected macaques for understanding HIV infection in humans. In addition to PMPA, other compounds that have shown activity in SIV-infected macaques include d4T, ddI, and hydroxyurea, among others.

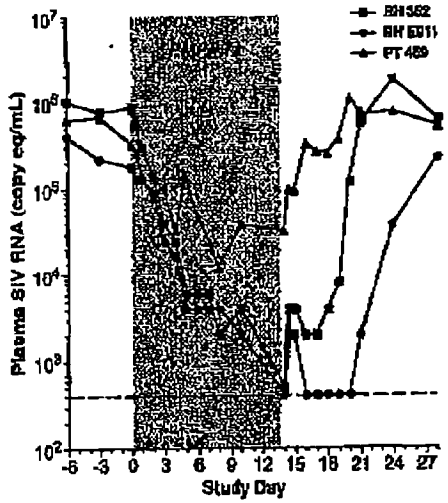

FIGURE 9 The effect of therapy with the antiviral drug PMPA on plasma viral RNA levels during chronic SIV infection for three macaques. Macaque RH 352 and RH 911 were inoculated with SIVsmE543-3 and macaque PT 459 was inoculated with SIVsmE660 (Nowak *et al.*, 1997). The shaded area indicates the period of drug treatment. Note the rapid decline in plasma viral RNA levels during the 14 days of treatment and the rapid rebound to pretreatment values after withdrawal of drug treatment.

C. Treatment of Acute SIV Infection

A variety of different treatment approaches have been evaluated for their ability to impact acute SIV infection. Both immunological and pharmacological approaches have been tried, often in studies designed to test the ability of a given regimen to mediate postexposure prophylaxis, i.e., to prevent the establishment of persistent pathogenic infection or modify the subsequent course of infection by treatment begun after exposure to infectious virus. Passive transfer of SIV immune globulin has been shown to modulate viral replication during primary infection (Haigwood *et al.*, 1997). Interestingly, the apparent effects of the immune globulin infusion persist long after circulating levels of the infused antibody had declined to below the threshold of detection. Treated animals showed lower levels of circulating virus and prolonged survival relative to controls, suggesting the possibility of long-lasting effects as a consequence of modulation of primary infection.

1. Studies with SIVMne

In postinoculation treatment models, PMPA treatment begun shortly after inoculation was able to prevent establishment of persistent infection with SIVMne (Tsai *et al.*, 1995, 1998; Van Rompay *et al.*, 1998, 1999). Both the interval between inoculation and initiation of treatment and the duration of treatment affected effectiveness in preventing persistent infection (Tsai *et al.*, 1998). Even in instances where the establishment of persistent infection was not prevented by postinoculation PMPA treatment, viral replication levels and clinical course were impacted, including in studies involving postinoculation treatment of neonatal macaques (Van Rompay *et al.*, 1996).

The treatment of neonatal macaques also underscores a unique SIV/macaque system of great value in validating the feasibility for impacting a preventable form of infection, *i.e.*, perinatal infection. In an SIV/neonatal macaque model of perinatal infection, PMPA has been shown to be capable of preventing the establishment of persistent infection, even using as few as two doses, bracketing the period of virus exposure (Van Rompay *et al.*, 1999).

These studies also underscore the potential of treatments impacting viral replication during primary infection to fundamentally modulate the subsequent pattern of viral replication and pathogenesis, including long-lasting effects manifested well after discontinuation of the treatment. This suggests that such treatment may induce a basic change in the dynamics of the relationship between the virus and the host, perhaps with regard to facilitating the development of immune responses capable of achieving long-term suppression of the virus. Studies in these types of postinoculation treatment models may usefully inform vaccine development efforts.

2. Studies with HIV-2

Transient postinoculation antiretroviral treatment has been shown to produce sustained, long-term impact on viral replication patterns, pathogen-

esis, clinical course, and survival in a different experimental model. As described above, infection of pigtail macaques with HIV-2/287 typically results in rapid, virtually complete destruction of the CD4+ T-cell population, with development of AIDS and death. Postinoculation treatment of HIV-2_{287}-infected animals with the reverse transcriptase inhibitor d4T produced lowered circulating viral loads, with persistent modulation of viral load even after drug treatment was discontinued (Watson *et al.*, 1997a). This effect was associated with prolonged survival in the treated animals compared to identically inoculated, untreated controls.

Like HIV-2_{287} of pigtail macaques, SHIV KU2 infection of rhesus macaques results in rapid, virtually complete destruction of the CD4+ T-cell population (Joag *et al.*, 1998). PMPA treatment, begun 1 week postinoculation, at a time when plasma SIV levels were in excess of 10^7 copy Eq/ml, with widely disseminated infection, was nevertheless capable of fundamentally altering the virus/host relationship. Although treatment did not result in clearance of the infection, following discontinuation of drug treatment plasma virus levels did not rebound to levels seen in the postacute phase of infection in untreated animals. Rather, circulating virus levels fluctuated at greatly reduced values, ranging from undetectable to peak values that were still orders of magnitude lower than those seen in untreated animals. This reduced level of viral replication, reflected by reduced levels of viremia, was not associated with the depletion of CD4+ T cells that is hallmark of pathogenic SHIV KU2 infection, through more than a year of follow-up after drug discontinuation. The two studies described above underscore the power of macaque infection models for studies of pathogenesis questions directly relevant to critical issues in human HIV infection, in this instance, achievement of host control of infection in the absence of continual lifelong antiretroviral therapy (Watson *et al.*, 1997a).

VI. Summary

As presented in this review, there are a number of different models of both natural and experimental infection of monkeys with primate lentiviruses. There are numerous different viruses and multiple different monkey species, making for a potentially large number of different combinations. The fact that each different combination of virus isolate and host macaque species may show different behavior underscores the need to understand the different models and their key features. On the one hand, this diversity of systems underscores the need to provide some standardization of the systems used for certain kinds of studies, such as vaccine evaluations, in order to facilitate the comparison of results obtained in different experiments, but in essentially the same experimental system. On the other hand, the rich diversity of different systems, with different features and behaviors, repre-

sents a tremendous resource, among other things allowing the investigator to select the system that best recapitulates particular aspects of human HIV infection for study in a relevant nonhuman primate model. Such studies have provided, and may be expected to continue to provide, important insights to guide HIV treatment and vaccine development in the future.

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Antiretroviral Drug Studies in Nonhuman Primates: a Valid Animal Model for Innovative Drug Efficacy and Pathogenesis Experiments

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Abstract

Several nonhuman primate models are used in HIV and AIDS research. In contrast to HIV-1 infection of chimpanzees, infection of macaque species with simian immunodeficiency virus (SIV) isolates results in a disease (simian AIDS) that shares many similarities with HIV infection and AIDS in humans. Although each animal model has its limitations and can never completely mimic HIV infection of humans, a carefully designed study allows experimental approaches, such as the control of certain variables, that are not feasible in humans, but that are often the most direct way to gain better insights in disease pathogenesis and provide proof-of-concept for novel intervention strategies. In the early days of the HIV pandemic, nonhuman primate models played a relatively minor role in the anti-HIV drug development process. During the past decade, however, the development of better virologic and immunologic assays, a better understanding of disease pathogenesis, and the availability of better drugs have made these animal models more practical for drug studies. In particular, nonhuman primate models have played an important role in demonstrating: (i) preclinical efficacy of novel drugs such as tenofovir; (ii) the benefits of chemoprophylaxis, early treatment and immunotherapeutic strategies; (iii) the virulence and clinical significance of drug-resistant viral mutants; and (iv) the role of antiviral immune responses during drug therapy. Comparison of results obtained in primate models with those observed in human studies will lead to further validation and improvement of these animal models. Accordingly, well-designed drug studies in nonhuman primates can continue to provide a solid scientific basis to advance our scientific knowledge and to guide future clinical trials. (AIDS Reviews 2005;7:67-83)

Key words

Macaque. Monkey. Prophylaxis. Chemotherapy. Resistance.

Introduction: the need for an appropriate animal model

An increasing arsenal of anti-HIV drugs is currently being used, and many novel candidates are continuously being developed[1]. The main anti-HIV drugs that have been approved or are being developed target several key steps or enzymes in the viral replication cycle: attachment, fusion, reverse transcriptase (RT), integrase or protease. During recent years, combination therapy of these compounds, so-called highly active antiretroviral therapy (HAART), has led to major improvements in the clinical management of HIV-infected people[2]. Despite this considerable success, there is no reason for complacency as long-term administration of these drugs is associated with problems of cost, toxicity, compliance, and drug resistance. Accordingly, the quest for better antiviral drug regimens continues. The ideal antiviral drug regimen would be one that induces strong and persistent suppression of virus replication, gives prolonged immunologic and clinical benefits without toxicity, can be administered at infrequent dosage intervals, is affordable and easy to store, and can thus benefit the greatest number of HIV-infected people, including those in developing countries.

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The pipeline that new drug candidates need to cross between the first demonstration of *in vitro* antiviral effects and approval for clinical use is tedious, time-consuming, and very expensive. Most compounds that inhibit virus replication *in vitro* are never further developed (due to lack of resources), or they fail in preclinical testing or clinical trials due to unfavorable pharmacokinetics, toxicity, or insufficient antiviral efficacy.

A confounding obstacle in the drug development process is that many drugs have already been approved for HIV-infected patients. It is considered unethical to treat "control" groups with anything less than the currently available "gold standard" of combination therapy. Therefore, the efficacy of new drugs is now often evaluated by including the compound as part of a combination regimen, often in patients failing currently available HAART regimens, who may have existing drug-resistance mutations, low CD4+ cell counts, or poor adherence. Thus, the response in such "worst-case scenario" patients may underestimate the potency of the drug for treatment-naive patients. These dilemmas underscore the need for an evaluation of the role of animal models in the drug development process. Appropriate animal models that allow rapid evaluation of the efficacy and toxicity of antiviral compounds can assist in sorting out those drugs which are promising and deserve to enter human clinical trials first, from those drugs that should probably be discarded[3].

While murine and feline models are appropriate for initial screening, further testing is best done in nonhuman primate models that better resemble HIV infection of humans. Nonhuman primates are phylogenetically the closest to humans. The similarities in physiology (including drug metabolism, placentation, fetal and infant development, etc.) and immunology allow a more reliable extrapolation of results obtained in primate models to clinical applications for humans. While chimpanzees can be infected with HIV-1, this animal model is not practical due to the low availability, high price, low viral virulence, and ethical issues[4,5]. Many nonhuman primate species in Africa are naturally infected with simian immunodeficiency virus (SIV) strains; despite persistent high-level virus replication, these natural hosts do not develop disease, possibly because infection is associated with little immune activation[6,7]. In contrast however, infection of non-natural hosts, such as macaques, with virulent SIV isolates results in a disease which resembles human AIDS (including generalized immune activation, CD4+ T-cell depletion, opportunistic infections, weight loss and wasting), and the same laboratory markers can be used to monitor disease progression[8]. Compared to HIV infection of humans, infection of macaques with virulent SIV or simian-human immunodeficiency virus (SHIV) isolates results in an accelerated course, as most animals develop clinical disease within one to three years. Similar to observations in HIV-infected human infants, the disease course in newborn macaques following inoculation with virulent SIV strains is usually accelerated[9,10]. It is important, however, to remember that SIV or SHIV infection of macaques is not necessarily fatal, as there are many attenuated or nonpathogenic virus isolates which give transient or low-level viremia, and slow or no disease. This wide spectrum of infection outcomes makes this model suitable to assess how genetic changes in the virus (e.g. drug-resistance mutations) affect viral virulence.

Primate models are powerful tools in many areas of HIV research. In addition to allowing investigators to unravel virus-host interactions during disease pathogenesis and to test vaccines[8], macaques allow us to model the different aspects of antiviral drug treatment, including pharmacokinetics, toxicity, and antiviral efficacy. The balance among all these *in vivo* interactions (which is impossible to model accurately *in vitro*) determines the long-term clinical usefulness of the antiviral drug (Fig. 1).

Besides being a test system for preclinical screening of novel drug regimens, an animal model can also be used to test hypotheses that are difficult or impossible to explore in humans. By manipulating certain variables (e.g. the initiation of drug treatment relative to virus inoculation, duration of treatment, the age of the animals, the virulence and drug susceptibility of the virus inoculum, the status of the immune system), investigators can design studies to address very specific questions. As discussed further in this review, examples of this are studies focused on evaluating chemoprophylaxis, the *in vivo* virulence and clinical implications of drug-resistant viral mutants, and the role of antiviral immune responses on antiviral drug efficacy.

Macaque species and virus isolates used in antiviral drug studies

Anti-HIV drug studies in macaques generally used rhesus macaques *(Macaca mulatta)* or cynomolgus macaques *(M. Fascicularis)*[11]. The SIV isolates usually belonged to a few groups, in particular SIVmac, SIVsmm and SIVmne. Because the polymerase region of these SIV isolates has about 60% and 85% amino acid homology to HIV-1 and HIV-2, respectively, SIV is susceptible to many of the same nucleoside RT inhibitors (NRTI; e.g. zidovudine), nucleotide RT inhibitors (tenofovir, adefovir), integrase and protease in-

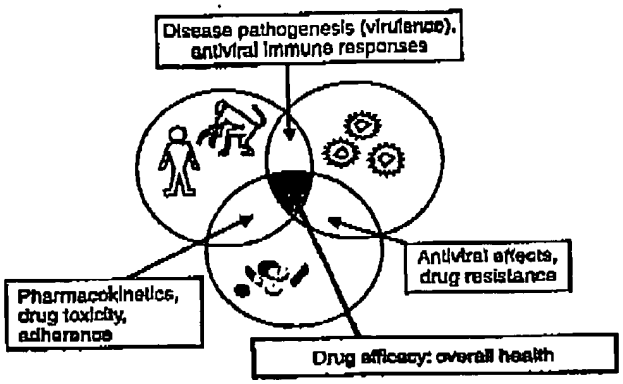

Figure 1. *Overall outcome of antiviral drug treatment. The ultimate goal of drug treatment is to improve the overall health of the host and indefinitely delay disease progression. This outcome is determined by many interactions between the virus, the host, and the antiviral drugs, most of which cannot be mimicked appropriately by in vitro studies. Animal models allow us to control and manipulate certain variables through experimental approaches that are not feasible in humans (such as experimental inoculation of animals with drug-resistant mutants, or in vivo depletion of certain immune cells), but that are often the most direct way to address certain questions regarding antiviral drug treatment.*

hibitors[12-16]. Due to their CCR5 chemokine coreceptor usage, SIV isolates are also susceptible to CCR5-targeting entry inhibitors[17]. Some compounds, however, including nonnucleoside RT inhibitors (NNRTI) such as nevirapine and efavirenz, are active only against HIV-1 and not against HIV-2 or SIV[18]. The construction of infectious SIV/HIV-1 chimeric viruses, in which the RT gene of SIV was replaced by its counterpart of HIV-1 (so called RT-SHIV), has been proven useful to evaluate NNRTI in primate models[19-23]. Other SHIV have been constructed and contain the envelope region (so called env-SHIV) or other genes of HIV-1. Many env-SHIV are attenuated. Most pathogenic env-SHIV such as SHIV-89.6P, while useful to address specific questions, have the limitation that their disease pathogenesis (including CXCR4 coreceptor usage and very rapid CD4+ cell depletion) is different from the typical course seen with HIV and SIV infection[24]. Currently available CCR5-using env-SHIV (such as SHIV-SF162P)[25] have the limitation that, after the initial peak of viremia, many untreated animals are able to suppress viremia to undetectable levels; while these isolates are useful to test prophylactic or early post-infection interventions, this large variability in chronic viremia set-point and disease outcome makes them less practical for testing antiviral drug efficacy during chronic infection, especially with limited animal availability. Accordingly, SIV is in general a more appropriate and practical model to test anti-HIV strategies[26,27].

Development of primate models: from initial obstacles to validation

During the first decade of the HIV pandemic, the role of nonhuman primate models in testing anti-HIV drugs was rather limited. Although SIV is susceptible to many anti-HIV drugs *in vitro*, many initial drug studies in macaques were not very successful in demonstrating *in vivo* efficacy[3,28]. Several factors are responsible for these observations. Most drugs that were available at that time had complicated dosage regimens (e.g. a short half-life necessitating frequent administration) or problems of toxicity and were thus not suitable for long-term administration. The time course of SIV disease progression in juvenile and adult macaques is highly variable as the asymptomatic period can range from months to years; it was therefore hard to determine whether a small difference in clinical outcome was due to host factors or to the drug treatment, especially with only relatively small numbers of animals and short-term treatment regimens[29]. In retrospect, another important reason for the poor efficacy results of the initial drug studies was that at that time the role of antiviral immune responses in determining antiviral drug efficacy was not

recognized. Untreated macaques infected with virulent isolates such as SIVmac251 have higher viremia, lower cell-mediated antiviral immune responses, and a more rapid disease course than HIV-infected humans[30]. As discussed further in this review, an antiretroviral drug becomes less effective in suppressing viremia without the assistance of effective antiviral immune responses. As the drugs available at that time were not very potent in suppressing viremia in HIV-infected humans, it is now no surprise that they were even less effective in suppressing viremia in immunodeficient SIV-infected macaques. Finally, sensitive assays to accurately quantitate viremia were not available at that time.

Many of these problems have been solved in the past decade. Sensitive assays, similar to those used to monitor HIV infection of humans, have been developed to monitor virus replication in SIV-infected macaques, including quantitative viral RNA assays[31-33]. The development of a pediatric SIV model has also been very useful, as the more uniformly rapid disease course (~ 3 to 4 months) observed in infant macaques infected with virulent SIV isolates permits evaluation of drug efficacy, including viremia and disease-free survival, in a relatively short time[29,34,35]. Infant macaques are also easier to handle for drug administration and require less drug, which is useful especially for compounds that are initially very expensive to produce in test quantities. The first report on the RT inhibitor tenofovir (9-[2-(R)-(phosphonomethoxy)propyl]adenine; PMPA) in 1995 was a milestone in validating this animal model because it was the first compound found to be highly effective against SIV infection[34,36]. The strong therapeutic benefits observed with tenofovir in the monkey studies have been predictive of tenofovir's efficacy in HIV-infected humans, and have contributed to its clinical development[37-39]. Altogether, these developments over the past decade have sparked further interest in using nonhuman primate models for antiretroviral drug studies.

Drug studies in nonhuman primates: overview and lessons learned

Pharmacokinetics and toxicity

Macaques, which are similar in physiology and metabolism to humans, have been very useful for studying the toxicity and pharmacokinetics of antiviral drugs, including the effects of pregnancy and drug transfer across the placenta and into breast milk[40-46]. While most studies used short-term drug administration (in the order of days to weeks), studies with tenofovir have also assessed the safety of prolonged treatment (> 1 to 10 years), starting at birth and continuing throughout adulthood, including pregnancy[47]. These studies found that prolonged daily treatment with a high dose of tenofovir resulted in a Fanconi-like syndrome (proximal renal tubular disorder) with bone pathology, while short-term administration of relatively high doses and prolonged low-dose regimens were safe[47]. Such long-term studies in primates are very relevant as they mimic life-long treatment of HIV-infected humans.

Prophylaxis: prevention of infection

Many studies in nonhuman primates have focused on investigating whether drug administration starting near the time of virus inoculation could prevent infection. Prevention of infection is traditionally considered as the complete absence of any viral or immunologic evidence of infection; however, the development of more sensitive techniques (including DNA PCR, viral RNA quantitation) has sometimes resulted in transient detection of low-level signs of infection, usually within the first months after virus inoculation[48,49]. Accordingly, for the purposes of this review, prophylaxis is defined as "protection against persistent infection", with persistent infection being defined as "persistent viremia or persistently detectable virus-specific immune responses".

A few studies in macaque models have evaluated the efficacy of antiviral compounds as topical microbicides against mucosal infection; topical high-dose administration of a number of compounds protected adult macaques against intravaginal or intrarectal SIV or SHIV infection at varying rates of efficacy[50-68].

Most studies have used systemic drug administration to try to prevent infection. Early studies, which mostly used zidovudine (AZT), were not very effective in preventing infection, but a likely reason for this was the high dose of virus used in these experiments[57-61]. In subsequent studies, when a lower dose of virus was used to inoculate animals, administration of several drugs (including zidovudine, adefovir (PMEA), tenofovir (PMPA) and 3'-fluorothymidine) starting prior to or at the time of virus inoculation was able to prevent virus infection[48,49,62-69]. Very few compounds have been shown to prevent infection when treatment was started after virus inoculation: i.e. tenofovir, BEA-005, and GW420867. A combination of the timing and duration of drug administration was found to determine their success rate[21,26,53,70-72]. Of these three compounds, tenofovir was effective following virus inoculation by different routes (intravenous, oral, intravaginal, intrarectal), and is currently the only one ap-

proved for therapeutic use in humans; BEA-005 and GW420867 are no longer in clinical development.

The demonstration that antiviral drugs can prevent infection in macaques has provided a solid scientific rationale to administer anti-HIV drugs to humans following exposure to HIV in several clinical settings. Antiviral drugs are now recommended, usually as combination regimens, to prevent HIV infection following occupational exposure (e.g. needlestick accidents of health care workers) and non-occupational exposure (e.g. sex or injection-drug use)[73,74]. Similarly to the animal studies, transient viremia has been described in some humans receiving postexposure prophylaxis[75].

Because an efficacious HIV vaccine has so far not been identified, tenofovir's prophylactic success in the macaque models has sparked clinical trials to investigate whether uninfected adult persons who engage in high-risk behavior will have a lower infection rate by taking tenofovir once daily. The ethical controversies surrounding these trials, which are being held at several international sites and target different high-risk populations, are reviewed elsewhere[76].

Antiviral drugs, especially zidovudine and nevirapine, have played a very important role in the prevention of mother-to-infant transmission of HIV, including in developing countries[77-79]. To counteract potential problems of drug-resistance mutations that are induced by the nevirapine regimen in women in developing countries[80], the promising data of a two-dose tenofovir regimen in the newborn macaque model[49,54] have spurred interest to test the feasibility of a two-dose tenofovir regimen to reduce perinatal HIV transmission (PACTG-394 and HPTN-057).

Therapy: treatment of infection

Many studies in the macaque model have demonstrated that, even when infection was not prevented, early drug treatment delayed or reduced the peak of acute viremia that occurs during the first weeks of infection, enhanced antiviral immune responses, and delayed disease progression[16,19,21,29,57,59,60,68,81-94]. These same benefits of early treatment have now been confirmed in human studies[95-100].

When macaques were started on short-term drug regimens during the stage of acute viremia, the outcome once treatment was withdrawn depended on the virus isolate. With pathogenic env-SHIV isolates, short-term suppression of acute viremia was usually effective to induce strong antiviral immune responses that controlled virus replication and delayed disease for an extended time in the absence of drug treatment[16,90,101]. In contrast, with highly virulent SIV isolates (such as SIVmac251), viremia usually increased again once short-term drug treatment was stopped, similarly to what is observed in most HIV-infected humans[28,27,94,102-105].

Macaque studies have also investigated the effects of antiviral therapy on established, chronic SIV infection (i.e. after the acute viremia stage), and the often disappointing results have puzzled researchers for a long time. Initial studies with zidovudine were not very successful in reducing viremia once SIV infection was established[29,62,106]. As selection for zidovudine-resistant viral mutants was slow[107], these data are consistent with the relative weakness of zidovudine monotherapy compared to newer compounds. Lamivudine (3TC) and emtricitabine ((-)-FTC) treatment of SIVmac251-infected infant macaques also had little effect on viremia and disease progression. However, there was rapid emergence of drug-resistant mutants with the M184V mutation in RT, suggesting that drug levels were sufficient to inhibit replication of wild-type virus[108]. The CCR5 inhibitor CMPD 167 reduced viremia fourfold to 200-fold in chronically SIV-infected macaques, but in some animals this effect was transient[17]. Similarly, efavirenz treatment led to reduced viremia in RT-SHIV infected animals, and selection for drug-resistant mutants led in some animals to viral rebound[23]. The integrase inhibitor L-870812 reduced viremia in SHIV-89.6P-infected macaques if initiated during early infection (before CD4+ cell depletion)[18]. In most studies, tenofovir has been highly effective to reduce established viremia[34,109-112]. During prolonged tenofovir therapy, the emergence of viral mutants with reduced *in vitro* susceptibility did not always lead to a rebound in viremia as some animals maintained low viremia[34,113]. However, there have been reports where tenofovir therapy was not effective in suppressing viremia despite the presence of drug-susceptible virus at the onset of treatment[38,101,109,112,114], suggesting that antiviral drug therapy is more complex than just a matter of having sufficient drug levels and susceptible virus. As discussed below, a growing body of evidence obtained from monkey studies creates a picture of drug therapy in which the efficacy of a drug regimen to reduce viremia is the combined result of several factors: (i) direct inhibitory activity of the drug(s) against the virus, determined by pharmacokinetic and pharmacodynamic factors; (ii) drug resistance (including likelihood of emergence, level of reduced susceptibility, effect of mutations on viral replication fitness and virulence); and (iii) the status of the host immune system (including antiviral immune responses). Primate studies

have provided valuable insights into these interactions. The demonstration of tenofovir's antiviral efficacy in SIV-infected macaques has sparked many other drug studies in this animal model. Tenofovir-containing regimens have been used to gain a better understanding of disease pathogenesis and drug therapy, and to test additional intervention strategies. While SIV infection leads to rapid depletion of CD4+ T-cells from gut-associated lymphoid tissue (GALT) and gastrointestinal dysfunction[115-117], early tenofovir therapy was found to decrease mucosal virus levels and restore the CD4+ T-cell population in GALT; this was associated with up-regulation of growth factors and genes involved in repair and regeneration of the mucosal epithelium[118,119]. Combination treatment of SIV-infected macaques with tenofovir and two protease inhibitors (indinavir and nelfinavir) was found to improve immune responses against other organisms such as mycobacterium[120]. The macaque model has also been used to investigate the viral reservoirs during drug treatment: SIV-infected pigtailed macaques treated with tenofovir and emtricitabine were found to have viral reservoirs in resting CD4+ T-lymphocytes[121]. Similar to observations in humans, a combination of tenofovir, lamivudine, and Efavirenz was also found to be very effective to suppress viremia in RT-SHIV infected macaques, with no detectable emergence of drug-resistant mutants during treatment[122].

A number of studies have combined antiviral drug treatment with other strategies aimed at enhancing antiviral immune responses, so that when drug treatment was stopped, viremia was controlled better. These immunotherapeutic strategies include structured treatment interruption, the combination of antiviral therapy with active immunization with or without cytokine administration, and immune reconstitution via administration of autologous CD4+ T-cells collected prior to SIV infection[123-130]. A caveat in interpreting the data of several of these studies, however, is that the combination of a high dose of tenofovir, didanosine, and hydroxyurea in macaques is plagued by problems of pancreatic toxicity (probably due to didanosine), which sometimes results in life-threatening diabetes (including after drug withdrawal); the published reports do not discuss whether drug-related toxicity may have contributed to the mortality observed in some of these studies.

The value of primate models in studying drug resistance

Many individuals do not show the desired strong and persistent suppression of viral replication during HAART. Although other factors, such as compliance and individual variability in pharmacokinetics, also contribute to reduced efficacy of HAART, a major limiting factor is the emergence of viral mutants with reduced *in vitro* susceptibility to antiviral drugs (so called "drug-resistant mutants")[131]. Due to the high mutation rate of the virus, incomplete suppression of replication selects for viral variants with mutations that allow better replication in the presence of drugs. The relationship between drug adherence and the emergence of drug-resistant mutants is complex and seems to depend on the drug class[132].

While the correlation between specific mutations in the viral genome and *in vitro* reduced susceptibility has been well documented for most antiviral compounds, many unanswered questions remain regarding the exact clinical implications of these drug-resistant variants *in vivo*, and how to use this information to make treatment decisions. If drug resistance means that the drug is no longer effective, then it can just as well be withdrawn; but if there is still a partial response, then it will be counterproductive to discontinue drug administration unless better alternatives can be offered[133-135]. Many studies, including those utilizing drug interruptions, have demonstrated that HAART can still have therapeutic virologic and/or immunologic benefits even in the presence of drug-resistant virus, and this may be due to some residual drug activity and/or the altered pathogenesis of drug-resistant variants[136-145]. Thus, it is important to note that the terms "drug resistance" and "reduced susceptibility" are *in vitro* measures, and "drug resistance" does not necessarily imply that drug efficacy is completely abolished *in vivo*.

An important question about mutants with reduced *in vitro* susceptibility to drugs concerns the replicative fitness and virulence of such mutants in comparison to wild-type virus. Because the mutations that reduce susceptibility are at very low or undetectable frequency in the absence of drug treatment, these mutations are expected to reduce the ability of the virus to replicate. However, primary drug-resistance mutations are often followed by compensatory mutations to improve replicative fitness. So what is the final result? Are drug-resistant mutants attenuated in virulence (i.e. their ability to cause disease) to such extent that the purpose of continuing drug therapy could be to prevent reversion to the more virulent wild-type form?

Studies measuring *in vitro* replication kinetics of drug-resistant HIV mutants can never completely predict their *in vivo* virulence. *In vivo* virulence is determined by complex pharmacologic, viral and host factors (including many tissue- and cell-specific factors)

that are difficult to mimic *in vitro*, such as drug pharmacokinetics, primary and compensatory mutations (and their impact on replication fitness, but also on immunogenicity), cell tropism, and the complex role of the immune system (which supports virus replication, but at the same time also tries to contain it). Studies in the SIV-macaque model have demonstrated repeatedly that the correlation between *in vitro* markers (viral replication fitness, cell tropism, and cytopathogenicity) and *in vivo* measures (replication fitness, cell tropism, and virulence) is often weak as virus isolates that replicate well and are very cytopathogenic *in vitro* can be severely attenuated or have a different cell tropism following inoculation in macaques[147-149]. Thus, the extrapolation of results from *in vitro* growth kinetic studies to decisions affecting clinical management of HIV-infected patients should be performed with caution. Similarly, it has been difficult to correlate data of *in vitro* drug susceptibility assays (which can demonstrate small to large changes in susceptibility) with changes in antiviral efficacy *in vivo*[150].

Some information regarding the relative replication fitness and stability of drug-resistant HIV mutants *in vivo* can be gathered from case reports, such as those documenting primary infection with drug-resistant HIV-1, as well as those monitoring the reversion of drug-resistant virus to wild-type following discontinuation of drug treatment[144,151,152]. An animal model, however, allows approaches which are impossible in humans, but which are the most direct ways to study the clinical implications of drug-resistant virus: animals can be inoculated with drug-resistant viral mutants or their wild-type counterparts, and their replication fitness and virulence can be compared in drug-treated versus untreated animals.

Drug-resistance studies in the macaque model

Several methods have been used to generate drug-resistant SIV variants *in vitro*, including selection through serial passage as well as site-directed mutagenesis of molecular clones[22,153,154]. Only a few studies have evaluated the emergence of drug-resistant viral mutants in treated macaques. Treatment of RT-SHIV infected macaques with nevirapine or efavirenz gave rise to the emergence of mutations at codon 103 and 181 in RT, similar to observations in treated HIV-1 infected patients[22,23].

A zidovudine-treated SIVmac251-infected macaque developed a glutamine-to-methionine substitution at codon 151 of RT (Q151M), associated with high-level (> 100-fold) *in vitro* resistance to zidovudine[29,107]. Inoculation of the Q151M SIVmac isolate into naive newborn macaques demonstrated that this mutation did not significantly reduce viral replication and viral virulence: the Q151M mutation (which is the result of two base changes) was also very stable in the absence of zidovudine treatment[107]. This Q151M mutation has not been found in HIV-1 infected patients receiving zidovudine monotherapy, but has been found in HIV-1 infected patients receiving sequential or combination therapy with dideoxynucleoside analogues[155,156]. However, the Q151M mutation is found frequently in HIV-2 infected patients receiving NRTI therapy[157,158]. This latter observation indicates that, due to much sequence homology, HIV-2 and SIV use similar mutational pathways that are sometimes distinct from those of HIV-1.

Treatment of SIV-infected infant macaques with lamivudine (3TC) or emtricitabine ((-)-FTC) gave rise to the emergence of viral mutants with the expected M184V mutation in RT within five weeks of treatment[108]. The clinical implication of the M184V mutation was subsequently investigated by inoculating juvenile macaques with SIVmac239 clones having either wild-type sequence or the M184V mutation in RT (SIVmac239-184V). In comparison to wild-type virus, SIVmac239-184V was replication-impaired, based on virus levels one week after inoculation, and on the reversion of SIVmac239-184V to wild-type sequence in untreated animals. However, this reduced replication fitness was not sufficient to affect viral virulence, as animals inoculated with SIVmac239-184V and treated with emtricitabine (to prevent reversion) had similar viremia from two weeks after infection onwards, and the disease course and survival was indistinguishable from that of animals infected with wild-type virus[108]. In a different study, the M184V mutation did not revert in macaques inoculated with SIVmac239 containing both the M184V and E89G mutations; however, the M184V mutation in that study was engineered with two base changes in codon 184 (instead of the single base change that is normally seen during *in vitro* or *in vivo* selections)[159].

Long-term treatment of SIVmac251-infected macaques with tenofovir resulted in the emergence of virus with fivefold reduced *in vitro* susceptibility to tenofovir, associated with a lysine-to-arginine substitution at codon 65 (K65R) of RT[34,114]. Tenofovir also selects for the K65R mutation in HIV-1 RT[160-162]. The emergence of K65R in SIV was followed by additional RT mutations, which were likely to be compensatory mutations[34]. The emergence and distribution of K65R mutants is a complex process, with considerable variability among animals and among tissues[114]. The SIV macaque model has provided impor-

tant information regarding the clinical implications of K65R viral mutants during tenofovir treatment. Although some SIVmac251-infected animals show an increase in viremia following the emergence of K65R viral mutants, other animals continue to suppress viremia to low or undetectable levels for years (> 3 to 9 years)[34,113,163]. This success in persistently suppressing replication of the highly virulent SIVmac251 isolate with tenofovir monotherapy is unprecedented in this animal model[25,27]. To investigate whether this observation of suppressed viremia in some animals despite K65R virus was caused by an attenuating effect of the K65R mutation on viral replication fitness and virulence, two K65R SIV isolates were inoculated into new animals. In the absence of tenofovir treatment, the K65R SIV isolates were as fit and virulent as wild-type SIVmac251, based on their ability to induce high viremia and rapid disease (≤ 4 months) in newborn macaques[163]. However, in the presence of prolonged tenofovir treatment, the disease course was changed and two scenarios were possible: (i) K65R viremia was reduced and could become undetectable with prolonged disease-free survival (> 9 years)[113,163]; (ii) viremia remained high (> 10^6 to 10^7 RNA copies/mL plasma), but with continued tenofovir treatment, survival was increased significantly more than predicted based on viral RNA levels and CD4+ T-cell counts[25,113,163]. Such findings have not been observed with other antiviral drugs in the SIV-macaque model, which suggests that tenofovir treatment may have rather unusual interactions with the immune system. These observations instigated further *in vivo* experiments that identified a major role of the immune system in determining the efficacy of antiviral drug therapy to reduce viremia.

The role of the immune system on the efficacy of drug therapy

Viral kinetics during drug therapy depend on viral replication fitness, drug susceptibility of the virus, and drug potency[164-169]. When virus levels in plasma are reduced rapidly following the onset of drug therapy, the antiviral drugs are lauded for their potency, while the role of antiviral immune responses during drug therapy is less clear[168]. In this context, one is inclined to consider antiviral immune responses mostly as a backup plan to try to contain viremia whenever drug treatment is withdrawn or if drug-resistant virus would emerge[163]. Recently, however, a growing body of evidence from human and primate studies suggests that antiviral immune responses play a previously unrecognized role during drug therapy, which merits proper credit[18,25,113,143,167]. Drug studies in macaques have demonstrated the concept that the efficacy of antiviral drug therapy in reducing viremia is not only determined by the intrinsic potency of the drug in directly inhibiting virus replication, but is also strongly dependent on the status of the immune system[18,25,113]. In other words, antiviral drugs require the assistance of immune responses to reach full effectiveness in reducing viremia, both at the onset of treatment when the virus has wild-type susceptibility, as well as during prolonged treatment in the presence of drug-resistant mutants[113].

Several key studies using experimental depletion of CD8+ cells *in vivo* (through administration of anti-CD8 monoclonal antibody) are summarized in figure 2, and support the model shown in figure 3. When tenofovir treatment was started during acute viremia with wild-type SIVmac251, the efficacy of tenofovir to suppress acute viremia with wild-type SIVmac251 was significantly reduced in the absence of CD8+ cells[113]. These observations indicate that the otherwise rapid decline of productively infected cells (with half-life of ~ 1 to 2 days) after the onset of drug therapy is due to CD8+ cell-mediated killing or inhibition, rather than the natural death rate (as determined by the cytopathogenicity of the virus)[113]. In this model of drug therapy (Fig. 3), CD8+ cell-mediated antiviral immune responses contribute significantly to the antiviral effects of anti-HIV drugs, presumably by reducing the burst of virus replication in productively infected cells via cytolytic or noncytolytic pathways. In the absence of CD8+ cells, productively infected cells had a long half-life, suggesting that virulent SIV, during concomitant tenofovir treatment, is not as cytopathic as expected[113].

Even after the emergence of K65R SIV mutants, some tenofovir-treated animals were able to reach undetectable viremia[34,113]. A tempting explanation for this surprising observation, especially if seen in tenofovir-treated humans, would be to ascribe it to (i) a severe reduction in replication fitness caused by the K65R mutation (which, as discussed earlier, is not the case for K65R SIV isolates)[163], and/or (ii) sufficient residual inhibitory effect of tenofovir against these viral mutants (with ~ 5-fold reduced *in vitro* susceptibility). However, CD8+ cell-depletion experiments, which are not feasible in humans, revealed that the suppressed viremia of K65R SIV mutants during prolonged tenofovir treatment of macaques was largely due to strong CD8+ cell-mediated antiviral immune responses because, in the absence of CD8+ cells, (i) K65R viral mutants were very replication-competent, and (ii) tenofovir treatment alone was not sufficient to inhibit K65R SIV replication *in vivo* (Fig. 2)[113].

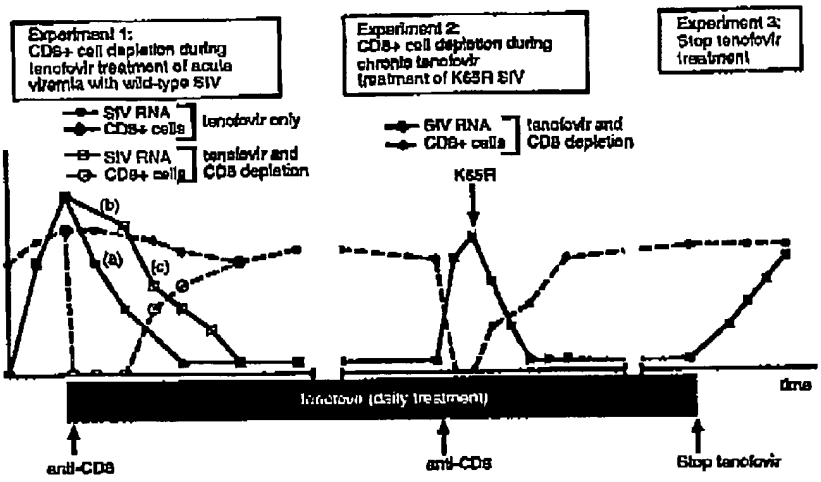

Figure 2. *Importance of CD8+ cells for the efficacy of tenofovir treatment: summary of CD8+ cell-depletion experiments. A schematic simplification of previously published data is presented*[113]. *In Experiment 1, animals were inoculated with wild-type virulent SIVmac251 and started on tenofovir therapy two weeks later. While untreated animals had persistently high viremia (not shown), animals started on tenofovir treatment (closed square) showed a rapid reduction of viremia (A), with estimated half-life of productively infected cells of 1 to 2 days in the presence of CD8+ cells. At the onset of tenofovir treatment, one group (open square and circle) was also depleted of CD8+ cells via administration of the anti-CD8 monoclonal antibody (cM-T807); in the absence of CD8+ cells, tenofovir-treated animals had little reduction in viremia (B), suggesting a half-life of productively infected cells of 4 to 6 days. When CD8+ cells became detectable, viremia was reduced rapidly with a half-life of 1 to 2 days (C). Despite the emergence of K65R mutants (with fivefold reduced in vitro susceptibility to tenofovir), some animals were able to reach undetectable viremia after prolonged tenofovir treatment*[113]. *In Experiment 2, when such chronically treated animals were depleted of CD8+ cells, viremia of K65R virus increased transiently and returned to baseline values upon return of CD8+ cells. Thus, tenofovir treatment alone was not sufficient to control viremia of K65R mutants in the absence of CD8+ cells. In Experiment 3, when prolonged tenofovir treatment was withdrawn, viremia of K65R virus increased slowly, demonstrating that CD8+ cell-mediated immune responses alone were not sufficient to maintain maximal suppression of viremia. Thus, both tenofovir and CD8+ cells were required for optimal suppression of viremia, both at the onset of therapy (when virus was still wild-type) as well as during prolonged therapy (when virus had reduced in vitro susceptibility and the K65R mutation in RT)*[113].

Further experiments demonstrated that continued tenofovir treatment was required to maintain suppression of K65R SIV replication because tenofovir withdrawal led to a slow increase in viremia (Fig. 2)[113]. Thus, both tenofovir and effective CD8+ cells were required to maximally suppress replication of virulent virus in this animal model. Because the anti-CD8 antibody depletes both CD8+CD3+ T-lymphocytes and CD8+CD3- natural killer (NK) cells, the relative contribution of these two cell populations and their antiviral effector mechanisms could not be identified in these experiments[113]. These observations of reduced viremia of K65R SIV mutants associated with improved antiviral immune responses in tenofovir-treated macaques are consistent with clinical observations of strong antiviral immune responses in HAART-treated HIV-1-infected people who have low-level viremia with drug-resistant virus[142,168]. Temporal variability in the strength of such immune responses may also be the direct cause of transient blips of viremia that are observed in many HAART-treated individuals[169,170]. Antiviral immune responses may thus also play a role in determining viral reservoirs in HAART-treated patients[171].

As mentioned previously, tenofovir treatment initiated during early stages of SIV infection was usually very effective in reducing viremia. In contrast, several studies documented that tenofovir therapy was not very effective in rapidly suppressing viremia, despite the presence of drug-susceptible virus at the onset of treatment, especially when tenofovir therapy was started later in infection, with more virulent isolates, and in animals with high viremia and immunodeficiency[35,101,109,112,114]. However, the rapid emergence of K65R virus that has been described in some of these studies is a reflection of strong selection pressure, and

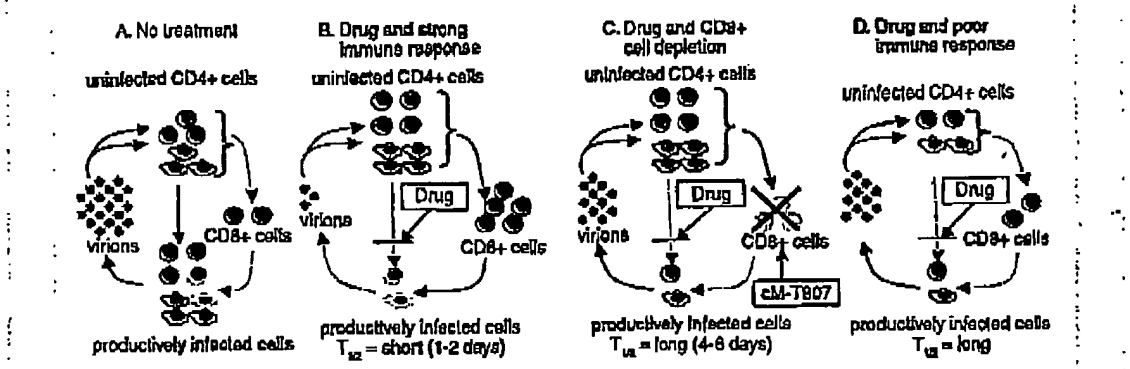

Figure 3. *Proposed model of drug and immune-mediated effects on virus replication. A: Without drug treatment, virulent virus can replicate to high titers because of high infection rates of CD4+ T-helper cells and antigen-presenting cells which are unable to provide sufficient assistance to CD8+ cell-mediated immune responses to contain virus replication. B: A potent drug regimen reduces the number of CD4+ T-helper cells and antigen-presenting cells that become newly infected. Potent CD8+ cell-mediated immune responses reduce the half-life, and thus the burst size of viral progeny, for those cells that already became infected. The combined antiviral activities of drug and antiviral CD8+ cells are efficient to induce and maintain low viremia, even after the emergence of drug-resistant viral mutants (as shown for tenofovir in the macaque model[114]). C: During artificial CD8+ cell depletion, productively infected cells survive longer and produce more progeny virus, resulting in higher viremia (see also Fig. 2)[113]. D: During immunodeficiency, the reduced function of antigen presenting cells and CD4+ T-helper cells results in insufficient assistance to antiviral CD8+ cells to remain active, especially at lower levels of viremia. Even when infection of new cells is reduced by an efficient drug regimen, the half-life of the productively infected cells is long, resulting in a slower decrease of viremia. Without sufficient immune restoration, the emergence of drug-resistant mutants is likely to lead to a rebound in viremia[16,25].*
Modified from reference 113.

indicates efficient inhibition of wild-type virus replication by the tenofovir regimen[25]. An integrase inhibitor was also found to be less effective in reducing viremia when initiated during late infection[16]. These data provide further support for this model in which antiviral immune responses assist anti-HIV drugs in reducing viremia. In the absence of effective antiviral immune responses, antiviral drugs face a more daunting task to control viremia as already infected cells survive longer and produce more viral progeny (Fig. 3D)[35,113]. Because virulent SIV isolates induce immune dysfunction at many stages of the immune response (including antigen presentation and CD4+ T-helper cell function[172,173]), CD8+ cell-mediated immune responses become inactive at lower levels of antigen, and thus it is less likely that viremia can be suppressed to low or undetectable levels, especially once drug-resistant mutants emerge[174-176]. This model in which both drugs and antiviral immune responses play a role in reducing viremia helps to explain the different patterns of viremia that are seen in drug-treated SIV-infected macaques and HIV-infected infants and adults[177,178]. Several main scenarios of models of viremia during drug therapy are presented in figure 4. Note, however, that an individual's pattern may shift to another one based on changes in drug regimen, the potential of immune restoration (including increased potency of antiviral immune responses), and the acquisition of additional drug resistance mutations (which can affect virulence and replication fitness). Even in an individual host, patterns of viral kinetics and turnover may vary among different tissues, based on tissue-specific differences in target cells, drug levels, and antiviral immune-effector mechanisms; this could explain observations of highly uneven distribution of SIV mutants in drug-treated macaques[114]. Such mechanisms of immune-mediated clearance of virus during drug therapy are probably not unique to lentiviruses, as a similar correlation has been described between the status of the immune system and clearance of hepatitis B virus following lamivudine treatment in patients with dual HIV and hepatitis B infection[179]. Despite this recent progress in better appreciating the role of antiviral immune responses during drug therapy, we need to acknowledge the big gaps that still remain in our knowledge of these antiviral immune responses. Direct *in vivo* ma-

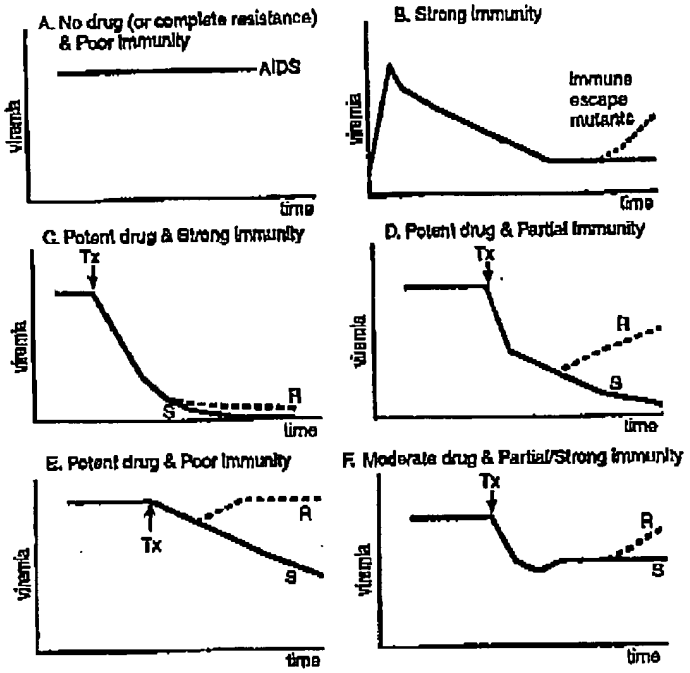

Figure 4. *Models of viremia during antiviral drug therapy: interaction of drugs and antiviral immune responses. Several scenarios are presented using different combinations of variables, including the strength of antiviral immune responses, the potency of the antiviral drug regimen against the virus, and the virulence and replication fitness of the virus. Tx indicates the start of drug treatment; R indicates the emergence of drug-resistant mutants with sufficient replication fitness, while S indicates viremia of wild-type virus (and/or drug-resistant mutants with severely reduced replication fitness). Intermediate levels of viral fitness are possible (not shown). "Potent drug" indicates a highly effective (single or combination) drug regimen that would completely prevent infection of new cells. A: Without effective antiviral immune responses and antiviral drugs (or in the presence of totally ineffective therapy due to complete drug resistance), viremia remains persistently high and leads to rapid disease. B: In the absence of anti-HIV drug therapy, some individuals are able to mount strong antiviral immune responses that initially control viremia, but usually are lost (due to progressive immune dysfunction and/or the emergence of immune escape mutants). C: Starting a potent drug regimen at a time of strong antiviral immune responses (e.g. during acute viremia) leads to rapid reduction of viremia; viremia can become and remain undetectable, even after the emergence of replication-fit drug-resistant virus (as observed in tenofovir-treated SIV-infected macaques[113]; see Fig. 2). D: Starting drug treatment at a moment of partial immunity (e.g. most HIV-infected patients with chronic infection) leads to a first phase of rapid decline in viremia, followed by phases of slower decline. These phases, generally believed to reflect distinct populations of infected cells[194], may alternatively also reflect antiviral immune responses that, without sufficient assistance of antigen-presenting cells or T-helper cells, become less active at lower levels of antigen[195]. In the absence of sufficient immune restoration, the emergence of drug-resistant virus or withdrawal of drug treatment is likely to lead to increased viremia. E: Without effective antiviral immune responses (e.g. SIV- or SHIV-infected macaques with severe immunodeficiency)[18,35,123], treatment with an otherwise highly potent drug does not result in rapid reduction in viremia, despite the presence of wild-type virus. Viremia can only continue to decrease if the drug is 100% effective in preventing infection of new cells and there is no emergence of drug-resistant mutants. F: With a partially effective drug regimen (or suboptimal levels of a potent drug), the reduction in viremia is limited because the relative increase in CD4+ cells provides more target cells for virus replication; as a result, viremia can stabilize at a lower level. Because wild-type virus can still replicate (albeit at reduced levels), the detection of drug-resistant mutants is delayed (e.g. zidovudine[69,107]).*

nipulations of the immune system (such as experimental depletions), which are often the best way to get a better understanding of *in vivo* antiviral immune mechanisms, can be performed in animal models, but are usually not feasible in humans. Instead, the need to rely on *in vitro* and *ex vivo* immune assays has the limitation that the currently available assays, especially when performed on peripheral blood, are not able to accurately grasp the variety, breadth, and strength of antiviral immune-effector mechanisms that control virus replication *in vivo*, especially in the lymphoid tissues and at mucosal sites[143,180-184].

It is important to note that the effects of antiviral immune responses during drug therapy are not mutually exclusive of the effects of reduced replication fitness of mutant virus and/or residual drug activity. In particular, even a relatively minor decrease in replication fitness, or a partial inhibition of virus replication by the drug regimen, can have a major impact on viremia if it provides more opportunity for effective antiviral immune responses to kill productively infected cells prior to the major viral burst. In contrast, in the absence of effective antiviral immune responses (such as during late-stage disease), a small difference in replication fitness may not translate into any significant difference in viremia and clinical outcome[108,113,185].

As mentioned previously, a surprising observation was that tenofovir-treated animals that maintained high viremia of K65R virus had prolonged disease-free survival, significantly more than predicted based on viral RNA levels and CD4+ T-cell counts[35,183]. This improved survival despite high viremia was only observed in the presence of tenofovir treatment, and has so far not been described for any other drugs in this animal model[107,108]. This prolonged survival despite high viremia in tenofovir-treated macaques is reminiscent of "discordant" or "paradoxical" results that have been described in HAART-treated HIV-infected adults and children, especially with regimens containing protease inhibitors. In such discordant patients, there is immunologic benefit (as measured by improved CD4+ T-lymphocyte counts and/or antigen-specific immune responses) and clinical benefits despite virologic failure[140-142,144,177,188-189]. The available data suggest that a combination of factors plays a role in such discordant results, including a decreased replicative fitness and T-cell activating ability of the drug-resistant mutants[135,138,144,146], an anti-apoptotic effect of protease inhibitors that preserves CD4+ T-cells[189], improved virus-specific cellular immunity[190], and direct antimicrobial properties of protease inhibitors[191,192]. Our study with tenofovir-treated SIV-infected macaques had the surprising finding that improved survival despite high viremia was even observed in animals in the absence of a significant immunologic response (based on standard immunologic parameters such as CD4+ T-cell counts and antibody responses to SIV and test antigens)[35,183]. Such clinical benefits would be difficult to detect in human studies as it requires years of follow-up, and without a good virologic and immunologic response, drug regimens would probably be changed in the meantime. As discussed elsewhere, it is unclear whether this phenomenon of prolonged disease-free survival in tenofovir-treated macaques with high viremia is due to residual antiviral activity of tenofovir against K65R virus in particular cell types (for example, antigen-presenting cells), potentially leading to relative preservation of innate immunity, or due to immunomodulatory effects that are independent of its antiviral effects, but that may partially protect the immune system against the deleterious effects of persistent virus replication and/or immune activation[35]. Tenofovir, which has many immunomodulatory effects in murine models[193], primed rhesus macaque cells for increased interleukin-12 secretion *in vitro*[194].

Such observations further highlight our relatively poor understanding of disease pathogenesis, and the need for further research to unravel the complex interactions between viral, host, and pharmacologic factors that determine (I) control of virus replication, and (II) overall clinical outcome. The data of these macaque studies also suggest that the criteria for changing treatment regimens that were established with older drug regimens (based on correlations between viral RNA levels, CD4+ cell counts and disease progression) may have to be modified for regimens that include newer drugs (such as tenofovir). Please note, however, that tenofovir-treated animals with high viremia, despite having improved survival, eventually still develop disease. Thus, the ultimate goal of antiviral therapy remains to inhibit virus replication maximally and restore the immune system, using regimens that are feasible with regard to safety, cost, and adherence.

Studies in SIV-infected macaques have shown that improvement of immunologic control of viremia is possible with adoptive transfer of autologous antigen-presenting cells, CD4+ T-helper cells, or other immunization strategies[124-130,195]. The studies with tenofovir in macaques have proven the concept that the combination of a potent drug regimen and good antiviral immune responses is able to induce long-term suppression of viremia and prolonged disease-free survival (> 3 to 9 years), even in the presence of mutants with reduced drug susceptibility[113]. Accordingly, these primate studies provide a strong scientific rationale to explore other strategies to boost or restore antiviral immune responses during antiviral therapy. The demonstration in SIV-infected macaques that antiviral immune responses already contribute significantly to rapidly reducing viremia immediately after the onset of drug therapy (Fig. 2) provides the scientific impetus to also explore the feasibility of starting immunotherapeutic strategies near to or simultaneously with the onset of antiviral drug therapy, instead of waiting until viremia has reached lower levels.

Conclusions

The development of better reagents and more sensitive virologic and immunologic assays, the discovery of more potent drugs, and a better understanding of disease pathogenesis have made nonhuman primate models a more practical and adaptable system (i) to rapidly evaluate novel prophylactic and therapeutic drug strategies, and (ii) to test hypotheses that cannot be mimicked appropriately by *in vitro* experiments and are difficult to explore in humans. The comparison and correlation of results obtained in monkey and human studies is leading to a growing validation and recognition of the relevance of this animal model. Although each animal model has its limitations, carefully designed drug studies in nonhuman primates can continue to advance our scientific knowledge and guide future clinical trials.

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Atty. Dkt. No. 029849-0203

CERTIFICATE OF MAILING BY "FIRST CLASS MAIL

Date of Deposit: April 12, 2004

I hereby certify that this correspondence is being deposited with the United States Postal Service as first class mail with sufficient postage on the date indicated above and is addressed to Commissioner for Patents, P.O. Box 1450, Arlington, VA 22313-1450.

Joy Day
Printed Name

Signature

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: VAILLANT, ANDREW *et al.*

Title: ANTIVIRAL OLIGONUCLEOTIDES TARGETING HIV

Appl. No. 10/661,099

Filing Date: September 12, 2003

Examiner: Unknown

Art Unit: 1614

AMENDMENT IN RESPONSE TO NOTICE UNDER 37 CFR §§1.821-825

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450
Mail Stop Missing Parts

Sir:

In response to the Notice to Comply With Requirements for Applications Containing Sequence Disclosures dated December 10, 2003, please amend the application as follows:

In the Specification:

Please amend the specification as shown:

Please delete paragraph [0068] and replace it with the following paragraph:

[0068] In particular embodiments, the oligonucleotide binds to one or more viral proteins; the sequence of the oligonucleotide (or a portion thereof, e.g., at least ½) is derived from a viral genome; the activity of an oligonucleotide with a sequence derived from a viral genome is not superior to a randomer oligonucleotide or a random oligonucleotide of the same length; the oligonucleotide includes a portion complementary to a viral sequence and a portion not complementary to a viral sequence; the sequence of the oligonucleotide is derived from a viral packaging sequence or other viral sequence involved in an aptameric interaction; unless otherwise indicated, the sequence of the oligonucleotide includes A(x), C(x), G(x), T(x), AC(x), AG(x), AT(x), CG(x), CT(x), or GT(x), where x is 2, 3, 4, 5, 6, ... 60 ... 120 **(SEQ ID NOS 27-36, respectively)** (in particular embodiments the oligonucleotide is at least 29, 30, 32, 34, 36, 38, 40, 46, 50, 60, 70, 80, 90, 100, 110, or 120 nucleotides in length or the length of the specified repeat sequence is at least a length just specified); the oligonucleotide is single stranded (RNA or DNA); the oligonucleotide is double stranded (RNA or DNA); the oligonucleotide includes at least one Gquartet or CpG portion; the oligonucleotide includes a portion complementary to a viral mRNA and is at least 29, 37, or 38 nucleotides in length (or other length as specified above); the oligonucleotide includes at least one non-Watson-Crick oligonucleotide and/or at least one nucleotide that participates in non-Watson-Crick binding with another nucleotide; the oligonucleotide is a random oligonucleotide, the oligonucleotide is a randomer or includes a randomer portion, e.g., a randomer portion that has a length as specified above for oligonucleotide length; the oligonucleotide is linked or conjugated at one or more nucleotide residues to a molecule that modifies the characteristics of the oligonucleotide, e.g. to provide higher stability (such as stability in serum or stability in a particular solution), lower serum interaction, higher cellular uptake, higher viral protein interaction, improved ability to be formulated for delivery, a detectable signal, improved pharmacokinetic properties, specific tissue distribution, and/or lower toxicity.

Atty. Dkt. No. 029849-0203

Please delete paragraph [000143] and replace it with the following paragraph:

[00143] Figure 37. (A) IC50 values generated from a plaque reduction assay conducted in VERO cells using HSV-1 (strain KOS). Infected cells are treated with increasing concentrations of REP 2006 (N40), REP 2028 (G40) **(SEQ ID NO: 21)**, REP 2029 (A40) **(SEQ ID NO: 20)**, REP 2030 (T40) **(SEQ ID NO: 23)**, and REP 2031 (C40) **(SEQ ID NO: 22)** to generate IC50 values. (B) HSV-1 PRA generated IC50 values of the following: N40 (REP 2006), AC20 **(SEQ ID NO: 24)** (REP 2055, TC20 **(SEQ ID NO: 25)** (REP 2056), or AG20 **(SEQ ID NO: 26)** (REP 2057).

Please delete paragraph [000199] and replace it with the following paragraph:

[00199] We monitored the ability of PS-ODNs of different sequences to interact with several viral lysates. In each case, a 20-mer PS-ODN is labeled at the 3' end with FITC as previously described herein. The PS-ODNs tested consisted of A20 **(SEQ ID NO: 12)**, T20 **(SEQ ID NO: 15)**, G20 **(SEQ ID NO: 13)**, C20 **(SEQ ID NO: 14)**, AC10 **(SEQ ID NO: 16)**, AG10 **(SEQ ID NO: 17)**, TC10 **(SEQ ID NO: 18)**, TG10 **(SEQ ID NO: 19)**, REP 2004 and REP 2017. Each of these sequences is diluted to 4nM in assay buffer and incubated in the presence of 1ug of HSV-1, HIV-1 or RSV lysate. Interaction is measured by fluorescence polarization.

Please delete paragraph [000200] and replace it with the following paragraph:

[00200] The profile of interaction with all sequences tested is similar in all viral lysates, indicating that the nature of the binding interaction is very similar. Within each lysate, the PS-ODNs of uniform composition (A20 **(SEQ ID NO:12)**, G20 **(SEQ ID NO:13)**, T20 **(SEQ ID NO:15)**, C20 **(SEQ ID NO:14)**) were the weakest interactors with A20 **(SEQ ID NO:12)** being the weakest interactor of these by a significant margin. For the rest of the PS-ODNs tested, all of them displayed a similar, strong interaction with the exception of TG10 **(SEQ ID NO:19)**, which consistently displayed the strongest interaction in each lysate (see figure 35).

Please delete paragraph [00302] and replace it with the following paragraph:

[00302] To determine if non-specific sequence composition has an effect on ON antiviral activity, several PS-ODNs of equivalent size but differing in their sequence composition were tested for anti-HSV1 activity in the HSV-1 PRA. The PS-ODNs tested were REP 2006 (N20), REP 2028 (G40)**(SEQ ID NO: 21)**, REP 2029 (A40) **(SEQ ID NO: 20)**, REP 2030 (T40) **(SEQ ID NO: 23)** and REP 2031 (C40) **(SEQ ID NO: 22)**.. The IC50 values generated from the HSV-1 PRA (see figure 37) show that REP 2006 (N40) was clearly the most active of all sequences tested while REP 2029 (A40) **(SEQ ID NO: 20)** was the least active. We also note that, all the other PS-ODNs were significantly less active than N40 with their rank in terms of efficacy being N40>C40 **(SEQ ID NO: 22)**>T40> **(SEQ ID NO: 23)** A40 **(SEQ ID NO: 20)**>>G40 **(SEQ ID NO: 21)**.

Please delete paragraph [00303] and replace it with the following paragraph:

[00303] We also tested the efficacy of different PS ODNs having varying sequence composition with two different nucleotides (see figure 37b). The PS-ODN randomer (REP 2006) was significantly more efficacious against HSV-1 than AC20 **(SEQ ID NO: 24)**(REP 2055), TC20 **(SEQ ID NO: 25)** (REP 2056) or AG20 **(SEQ ID NO: 26)** (REP 2057) with their efficacies ranked as follows: N40>AG**(20)(SEQ ID NO: 26)**>AC**(20)(SEQ ID NO: 24)**>TC**(20)(SEQ ID NO: 25)**. This data suggests that although the anti-viral effect is non-sequence complementary, certain non-specific sequence compositions (ie C40 **(SEQ ID NO: 22)** and N40) have the most potent anti-viral activity. We suggest that this phenomenon can be explained by the fact that, while retaining intrinsic protein binding ability, sequences like C40 **(SEQ ID NO: 22)**, A40 **(SEQ ID NO: 20)**, T40 **(SEQ ID NO: 23)** and G40 **(SEQ ID NO: 21)** bind fewer viral proteins with high affinity, probably due to some restrictive tertiary structure formed in these sequences. On the other hand, due to the random nature of N40, it retains its ability to bind with high affinity to a broad range of anti-viral proteins which contributes to its robust anti-viral activity.

Atty. Dkt. No. 029849-0203

Please delete Table 1 and replace it with the following Table:

TABLE 1 – DESCRIPTION OF OLIGONUCLEOTIDES

REP 1001	20mer from human automonously replicating sequence
SEQUENCE	T T G A T A A A T A G T A C T A G G A C (SEQ ID NO: 1)
PS	? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ?
REP 2001	22mer from HSV-1 origin of replication
SEQUENCE	G A A G C G T T C G C A C T T C G T C C C A (SEQ ID NO: 2)
PS	? ?
REP 3007	16mer from pUC19/pBR322 origin of replication
SEQUENCE	C T T G C G G T A T T C G G A A (SEQ ID NO: 3)
PS	? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ?
REP 2002	5mer randomer
SEQUENCE	N N N N N
PS	? ? ? ? ?
REP 2032	6mer randomer
SEQUENCE	N N N N N N
PS	? ? ? ? ? ?
REP 2003	10mer randomer
SEQUENCE	N N N N N N N N N N
PS	? ? ? ? ? ? ? ? ? ?
REP 2009	12mer randomer
SEQUENCE	N N N N N N N N N N N N
PS	? ? ? ? ? ? ? ? ? ? ? ?
REP 2010	14mer randomer
SEQUENCE	N N N N N N N N N N N N N N
PS	? ? ? ? ? ? ? ? ? ? ? ? ? ?
REP 2011	16mer randomer
SEQUENCE	N N N N N N N N N N N N N N N N
PS	? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ?
REP 2012	18mer randomer
SEQUENCE	N N N N N N N N N N N N N N N N N N
PS	? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ?
REP 2004	20mer randomer
SEQUENCE	N N N N N N N N N N N N N N N N N N N N
PS	? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ?
REP 2005	30mer randomer
SEQUENCE	N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N
PS	? ?
REP 2006	40mer randomer
SEQUENCE	N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N
PS	? ?
REP 2007	80mer randomer
SEQUENCE	N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N 60
PS	? ?
SEQUENCE	N N N N N N N N N N N N N N N N N N N N 80
PS	? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ? ?
REP 2008	120mer randomer
SEQUENCE	N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N 60
PS	? ?
SEQUENCE	N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N 120
PS	? ?

REP 2013 10mer randomer
SEQUENCE N N N N N N N N N N
no modification

REP 2014 20mer randomer
SEQUENCE N N N N N N N N N N N N N N N N N N N N
no modification

REP 2015 40mer randomer
SEQUENCE N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N
no modification

REP 2016 10mer random sequence
SEQUENCE T C C G A A G A C G (SEQ ID NO: 4)
PS ? ? ? ? ? ? ? ? ? ?

REP 2017 20mer random sequence
SEQUENCE A C A C C T C C G A A G A C G A T A A C (SEQ ID NO: 5)
PS ?

REP 2018 40mer random sequence
SEQUENCE C T A C A G A C A T A C A C C T C C G A A G A C G A T A A C A C T A G A C A T A (SEQ ID NO: 6)
? ?

REP 2019 10mer sequence centered around start codon of HSV-1 IE110 protein (NCBI accession # X04614)
SEQUENCE C C C C C [redacted] (SEQ ID NO: 7)
PS ? ? ? ? ? ? ? ? ? ?

REP 2020 20mer sequence centered around start codon of HSV-1 IE110 protein (NCBI accession # X04614)
SEQUENCE T A C G A C C C C C [redacted] (SEQ ID NO: 8)
PS ?

REP 2021 40mer sequence centered around start codon of HSV-1 IE110 protein (NCBI accession # X04614)
SEQUENCE T C C A G C C G C A T A C G A C C C C C [redacted] (SEQ ID NO: 9)
PS ?

REP 2024 40mer randomer
SEQUENCE N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N
PS ?
2-O Me ? ? ? ? ? ? ? ?

REP 2028 40mer randomer
SEQUENCE N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N N
PO/O ? ? ? ? ? ? ? ?

REP 2036 21mer commercially marketed antisense against CMV (vitravine/fomvirisen) SYNTHESIZED INTERNALLY
SEQUENCE G C G T T T G C T C T T C T T C T T G C G (SEQ ID NO: 10)
PS ?

REP 2036 © 21mer commercially marketed antisense against CMV (vitravine/fomvirisen) COMMERCIAL PRODUCT (cGMP)
SEQUENCE G C G T T T G C T C T T C T T C T T G C G (SEQ ID NO: 11)
PS ?

A20 20 mer
SEQUENCE A A A A A A A A A A A A A A A A A A A A -FITC (SEQ ID NO: 12)
PS ?

G20 20 mer
SEQUENCE G G G G G G G G G G G G G G G G G G G G -FITC (SEQ ID NO: 13)
PS ?

C20 20 mer
SEQUENCE C C C C C C C C C C C C C C C C C C C C -FITC (SEQ ID NO: 14)
PS ?

T20 20 mer
SEQUENCE T T T T T T T T T T T T T T T T T T T T -FITC (SEQ ID NO: 15)
PS ?

AC10 20 mer
SEQUENCE A C A C A C A C A C A C A C A C A C A C -FITC (SEQ ID NO: 16)
PS ?

AG10 20 mer
SEQUENCE A G A G A G A G A G A G A G A G A G A G -FITC (SEQ ID NO: 17)
PS ?

TC10 20 mer

Atty. Dkt. No. 029849-0203

REMARKS

Applicants believe that the present application is now in condition for allowance. Favorable reconsideration of the application as amended is respectfully requested.

The Examiner is invited to contact the undersigned by telephone if it is felt that a telephone interview would advance the prosecution of the present application.

Respectfully submitted,

Date: April 12, 2004

By _____

Foley & Lardner LLP
11250 El Camino Real
Suite 200
San Diego, CA 92130
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Facsimile: 858-792-6773

Wesley B. Ames
Attorney for Applicant
Registration No. 40,893

Should additional fees be necessary in connection with the filing of this paper, or if a petition for extension of time is required for timely acceptance of same, the Commissioner is hereby authorized to charge Deposit Account No. 50-0872 for any such fees; and applicants hereby petition for any needed extension of time.

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Atty. Docket No: 029849/0203

In re patent application of

VAILLANT, ANDREW et al.

Serial No: 10/661,099

Filed: September 12, 2003

For: ANTIVIRAL OLIGONUCLEOTIDES TARGETING HIV

STATEMENT TO SUPPORT FILING AND SUBMISSION IN ACCORDANCE WITH 37 C.F.R. §§ 1.821-1.825

Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450
Mail Stop Missing Parts

Sir:

In connection with a Sequence Listing submitted concurrently herewith, the undersigned hereby states that:

1. the submiseion, filed herewith in accordance with 37 C.F.R. § 1.821(g), does not include new matter;

2. the content of the attached paper copy and the attached computer readable copy of the Sequence Listing, submitted in accordance with 37 C.F.R. § 1.821(c) and (e), reepectively, are the same.

Respectfully submitted,

Jan. 28, 2004
Date

David M. Narkunas
Reg. No. 53,370

HARBOR CONSULTING IP SERVICES, INC.
1500A Lafayette Road, #262
Portsmouth, N.H.
800-318-3021

SEQUENCE LISTING

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JUTEAU, JEAN-MARC

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<223> this sequence may encompass 2-120 'ag' repeats

<400> 32
agagagagag agagagagag agagagagag agagagagag agagagagag agagagagag 60
agagagagag agagagagag agagagagag agagagagag agagagagag agagagagag 120
agagagagag agagagagag agagagagag agagagagag agagagagag agagagagag 180
agagagagag agagagagag agagagagag agagagagag agagagagag agagagagag 240

<210> 33
<211> 240

<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic oligonucleotide

<220>
<223> this sequence may encompass 2-120 'at' repeats

<400> 33
atatatatat atatatatat atatatatat atatatatat atatatatat atatatatat 60
atatatatat atatatatat atatatatat atatatatat atatatatat atatatatat 120
atatatatat atatatatat atatatatat atatatatat atatatatat atatatatat 180
atatatatat atatatatat atatatatat atatatatat atatatatat atatatatat 240

<210> 34
<211> 240
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic oligonucleotide

<220>
<223> this sequence may encompass 2-120 'cg' repeats

<400> 34
cgcgcgcgcg cgcgcgcgcg cgcgcgcgcg cgcgcgcgcg cgcgcgcgcg cgcgcgcgcg 60
cgcgcgcgcg cgcgcgcgcg cgcgcgcgcg cgcgcgcgcg cgcgcgcgcg cgcgcgcgcg 120
cgcgcgcgcg cgcgcgcgcg cgcgcgcgcg cgcgcgcgcg cgcgcgcgcg cgcgcgcgcg 180
cgcgcgcgcg cgcgcgcgcg cgcgcgcgcg cgcgcgcgcg cgcgcgcgcg cgcgcgcgcg 240

<210> 35
<211> 240
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic oligonucleotide

<220>
<223> this sequence may encompass 2-120 'ct' repeats

<400> 35
ctctctctct ctctctctct ctctctctct ctctctctct ctctctctct ctctctctct 60
ctctctctct ctctctctct ctctctctct ctctctctct ctctctctct ctctctctct 120
ctctctctct ctctctctct ctctctctct ctctctctct ctctctctct ctctctctct 180
ctctctctct ctctctctct ctctctctct ctctctctct ctctctctct ctctctctct 240

<210> 36
<211> 240

<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Synthetic
oligonucleotide

<220>
<223> this sequence may encompass 2-120 'gt' repeats

<400> 36
gtgtgtgtgt gtgtgtgtgt gtgtgtgtgt gtgtgtgtgt gtgtgtgtgt 60
gtgtgtgtgt gtgtgtgtgt gtgtgtgtgt gtgtgtgtgt gtgtgtgtgt 120
gtgtgtgtgt gtgtgtgtgt gtgtgtgtgt gtgtgtgtgt gtgtgtgtgt 180
gtgtgtgtgt gtgtgtgtgt gtgtgtgtgt gtgtgtgtgt gtgtgtgtgt 240

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